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(54) Title: TUMOUR MARKERS

(57) Abstract

A method of determining the immune response of a mammal to circulating tumour marker proteins is described in which a sample of bodily fluid, for example plasma or serum, is contacted with a panel of two or more distinct tumour marker antigens. The presence of complexes between the tumour marker antigens and any autoantibodies to the antigens present in the sample are detected and provide an indication of an immune response to a circulating tumour marker protein. The method is useful for the diagnosis of cancer, particularly for identifying new or recurrent cancer in an otherwise assymptomatic patient.

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TUMOUR MARKERS

The invention relates to methods of detecting or quantitatively measuring the immune response of a mammal to circulating tumour markers or tumour markers expressed on the surface of tumour cells, also to tumour marker antigens for use in these methods, to kits for performing the methods and to the use of these methods in the detection of cancer, in monitoring the progress of cancer, in detecting recurrent disease in cancer patients who have previously undergone anti-cancer treatment and in predicting the response of a cancer patient to a particular course of treatment.

The development and progression of cancer in a patient is generally found to be associated with the presence of markers in the bodily fluid of the patient, these "tumour markers" reflecting different aspects of the biology of the cancer (see Fateh-Maghadam, A. & Steilber, P. (1993) Sensible use of tumour markers. Published by Verlag GMBH, ISBN 3-926725-07-9). Tumour markers are often found to be altered forms of the wild type proteins expressed by 'normal' cells, in which case the alteration may be a change in primary amino acid sequence, a change in secondary, tertiary or quaternary structure or a change in post-translational modification, for example, abnormal glycosylation. Alternatively, wild type proteins which are up-regulated or over-expressed in tumour cells, possibly as a result of gene amplification or abnormal transcriptional regulation, may also be tumour markers.

Established assays for tumour markers present in

bodily fluids tend to focus on the detection of tumour markers which reflect tumour bulk and as such are of value late in the disease process, for example, in the diagnosis of metastatic disease. The most widely used of these markers include carcinoembryonic antigen (CEA) and the glycoprotein termed CA 15.3, both of which have been useful mainly as indicators of systemic disease burden and of relapse following therapy (Molina, R., Zanon, G., Filella, X. *et al*. Use of serial carcinoembryonic antigen and CA 15.3 assays in detecting relapses in breast cancer patients. (1995) *Breast Cancer Res Treat* 36: 41-48) These markers are of limited use earlier in the disease progression, for example in the screening of asymptomatic patients. Thus, in the search for tumour markers present in bodily fluid that are of use earlier in the disease process the present inventors have sought to identify markers which do not depend on tumour bulk *per se*.

Differences between a wild type protein expressed by 'normal' cells and a corresponding tumour marker protein may, in some instances, lead to the tumour marker protein being recognised by an individual's immune system as 'non-self' and thus eliciting an immune response in that individual. This may be a humoral (i.e B cell-mediated) immune response leading to the production of autoantibodies immunologically specific to the tumour marker protein. Autoantibodies are naturally occurring antibodies directed to an antigen which an individual's immune system recognises as foreign even though that antigen actually originated in the individual. They may be present in the circulation as circulating free autoantibodies or in the form of circulating immune complexes consisting

of autoantibodies bound to their target tumour marker protein.

As an alternative to the direct measurement or detection of tumour marker protein in bodily fluids, assays could be developed to measure the immune response of the individual to the presence of tumour marker protein in terms of autoantibody production. Such assays would essentially constitute indirect detection of the presence of tumour marker protein. Because of the nature of the immune response, it is likely that autoantibodies can be elicited by a very small amount of circulating tumour marker protein and indirect methods which rely on detecting the immune response to tumour markers will consequently be more sensitive than methods for the direct measurement of tumour markers in bodily fluids. Assay methods based on the detection of autoantibodies may therefore be of particular value early in the disease process and possibly also in relation to screening of asymptomatic patients, for example to identify individuals "at risk" of developing disease.

Tumour marker proteins observed to elicit serum autoantibodies include a particular class of mutant p53 protein, described in US Patent No. 5,652,115, which can be defined by its ability to bind to the 70 kd heat shock protein (hsp70). p53 autoantibodies can be detected in patients with a number of different benign and malignant conditions (described in US 5,652,115) but are in each case present in only a subset of patients. For example, one study utilizing an ELISA assay for detection of autoantibodies directed against the p53 protein in the serum of breast cancer patients reported that p53 autoantibodies were produced by 26% of patients and

1.3% of control subjects (Mudenda, B., Green, J. A., Green, B. *et al*. The relationship between serum p53 autoantibodies and characteristics of human breast cancer.(1994) *Br J Cancer* **69**: 4445-4449.). A second tumour marker protein known to elicit serum autoantibodies is the epithelial mucin MUC1 (Hinoda, Y. *et al*. (1993) *Immunol Lett*. **35**: 163-168; Kotera, Y. *et al*. (1994) *Cancer Res*. **54**: 2856-2860).

In most cancers resulting from a progressive accumulation of genetic alterations, such as breast cancer, the presence of tumour markers in bodily fluids reflects the development and progression of disease but no single marker on its own summates all clinically important parameters. For example, the characteristics of a marker useful for diagnosis of cancer may be quite different from markers which convey information about prognosis. Furthermore, in each clinical situation (i.e. diagnosis or prognosis) different markers may be required when dealing with primary cancer and secondary (metastatic) cancer and a different marker again may be required to provide a method of measuring the effectiveness of a particular course of treatment. Different clinical situations therefore require different biological markers and, as has been observed with p53, not all patients express the same set of tumour marker proteins. It is therefore difficult to envisage any one single tumour marker being universally applicable to all patients in all stages of disease.

It is an object of the present invention to provide an improved assay system for the detection of bodily fluids-borne tumour markers which is more generally useful in all patients and in a variety of

different clinical situations.

Accordingly, in a first aspect the invention provides a method of detecting the immune response of a mammal to circulating tumour marker proteins or tumour cells expressing said tumour marker proteins, which method comprises steps of:

(a) contacting a sample of bodily fluids from said mammal with a panel of two or more distinct tumour marker antigens;

(b) determining the presence or absence of complexes of said tumour marker antigens bound to autoantibodies present in said sample of bodily fluids, said autoantibodies being immunologically specific to said tumour marker proteins.

whereby the presence of said complexes is indicative of the immune response to circulating tumour marker proteins or tumour cells expressing said tumour marker proteins.

The method of the invention, which may be hereinafter referred to as a 'panel assay', utilises a panel of two or more tumour marker antigens to monitor the overall immune response of an individual to a tumour or other carcinogenic/neoplastic change. The method thus provides essentially a 'profile' of the immune response for that individual, indicating which tumour markers elicit an immune response resulting in autoantibody production. The method of the invention is preferred for the detection of an immune response resulting in the production of circulating free autoantibodies.

Because the assay method of the invention performed on a sample of bodily fluids taken from the patient it is essentially non-invasive and can be repeated as often as is thought necessary to build up a profile of the patient's immune response throughout the course of disease. As used herein the term 'bodily fluids' includes plasma, serum, whole blood, urine, sweat, lymph, faeces, cerebrospinal fluid or nipple aspirate. The type of bodily fluid used may vary depending upon the type of cancer involved and the use that the assay is being put to. In general, it is preferred to perform the method on samples of serum or plasma.

As will be illustrated in the Examples given below, the use of a panel of two or more tumour marker antigens to monitor autoantibody production is more sensitive than the use of single markers and gives a much lower frequency of false negative results. The actual steps of detecting autoantibodies in a sample of bodily fluids may be performed in accordance with immunological assay techniques known *per se* in the art. Examples of suitable techniques include ELISA, radioimmunoassays and the like. In general terms, such assays use an antigen which may be immobilised on a solid support. A sample to be tested is brought into contact with the antigen and if autoantibodies specific to the tumour marker protein are present in the sample they will immunologically react with the antigen to form autoantibody-antigen complexes which may then be detected or quantitatively measured. Detection of autoantibody-antigen complexes is preferably carried out using a secondary anti-human immunoglobulin antibody, typically anti-IgG or anti-human IgM, which recognise general features common to

all human IgGs or IgMs, respectively. The secondary antibody is usually conjugated to an enzyme such as, for example, horseradish peroxidase (HRP) so that detection of autoantibody/antigen/secondary antibody complexes is achieved by the addition of an enzyme substrate and subsequent colorimetric, chemiluminescent or fluorescent detection of the enzymatic reaction products.

The panel assay of the invention uses a panel of tumour marker-related antigens. The panel may be tailored to detect a particular cancer, or a cancer at a particular stage of development. The tumour marker antigens may be wild type or mutant tumour marker proteins isolated from samples of biological fluid from normal individuals or from cancer patients or from cell lines expressing the tumour marker protein or they may be full length recombinant tumour marker proteins, viral oncogenic forms of tumour marker proteins or antigenic fragments of any of the aforementioned proteins. The term 'antigenic fragment' as used herein means a fragment which is capable of eliciting an immune response.

The panel assay may be performed in a multi-well format in which each one of the two or more antigens is placed in a separate well of a multi-well assay plate or, alternatively, in a single-pot format in which the entire panel of antigens is placed in a single container. The panel assay may be performed in a qualitative format in which the objective is simply detection of the presence or absence of autoantibodies or in a quantitative format which provides a quantitative measurement of the amount of autoantibodies present in a sample.

Preferred markers for inclusion into the panel of

tumour marker antigens include the epidermal growth factor receptor-related protein c-erbB2 (Dsouza, B. *et al.* (1993) *Oncogene.* **8**: 1797-1806), the glycoprotein MUC1 (Batra, S. K. *et al.* (1992) *Int. J. Pancreatology.* **12**: 271-283) and the signal transduction/cell cycle regulatory proteins Myc (Blackwood, E. M. *et al.* (1994) *Molecular Biology of the Cell* **5**: 597-609), p53 (Matlashewski, G. *et al.* (1984) *EMBO J.* **3**: 3257-3262; Wolf, D. *et al.* (1985) *Mol. Cell. Biol.* **5**: 1887-1893) and ras (or Ras) (Capella, G. *et al.* (1991) *Environ Health Perspectives.* **93**: 125-131), including the viral oncogenic forms of ras which can be used as antigens to detect anti-ras autoantibodies, and also BRCA1 (Scully, R. *et al.* (1997) *PNAS* **94**: 5605-10), BRCA2 (Sharan, S. K. *et al.* (1997) *Nature.* **386**: 804-810), APC (Su, L. K. *et al.* (1993) *Cancer Res.* **53**: 2728-2731; Munemitsu, S. *et al.* (1995) *PNAS* **92**: 3046-50), CA125 (Nouwen, E. J. *et al.* (1990) *Differentiation.* **45**: 192-8) and PSA (Rosenberg, R. S. *et al.* (1998) *Biochem Biophys Res Commun.* **248**: 935-939). As aforementioned, the assays can be formed using tumour marker antigens which are forms of these proteins isolated from human bodily fluids or from cultured cells or antigenic fragments thereof or full length or truncated recombinant proteins or antigenic fragments thereof.

Preferably the tumour marker antigens are labelled with biotin so that they can easily be attached to a solid support, such as a multi-well assay plate, by means of the biotin/avidin or biotin/streptavidin interaction. Tumour marker antigens labelled with biotin may be referred to

herein as 'biotinylated' proteins. To facilitate the production of biotinylated tumour marker antigens for use in the assay methods of the invention, cDNAs encoding a full length recombinant tumour marker protein, a truncated version thereof or an antigenic fragment thereof may be expressed as a fusion protein labelled with a protein or polypeptide tag to which the biotin co-factor may be attached via an enzymatic reaction. A useful system for the expression of biotinylated fusion proteins is the PinPoint™ system supplied by Promega Corporation, Madison WI, USA. The present inventors have surprisingly found that with the use of biotinylated tumour marker antigens as antigens they are able to detect autoantibodies in a much higher percentage of patients than is observed using non-biotinylated antigen.

The assay method of the invention may be employed in a variety of different clinical situations such as, for example, in the detection of primary or secondary (metastatic) cancer, in screening for early neoplastic or early carcinogenic change in asymptomatic patients or identification of individuals 'at risk' of developing cancer (particularly breast cancer, bladder cancer, colorectal cancer or prostate cancer) in a population or asymptomatic individuals, in the detection of recurrent disease in a patient previously diagnosed as carrying tumour cells who has undergone treatment to reduce the number of tumour cells or in predicting the response of an individual with cancer to a course of anti-cancer treatment.

The assay method of the invention is suitable for detection of many different types of cancer, of which examples are breast, bladder, colorectal, prostate and ovarian. The assay of the invention may complement

existing methods of screening and surveillance. For example in the case of primary breast cancer it could be used to alert clinicians to biopsy small lesions on mammograms which radiographically do not appear suspicious or to carry out breast imaging or to repeat imaging earlier than planned. In the clinic, the assay method of the invention is expected to be more objective and reproducible compared to current imaging techniques (i.e. mammography and ultrasound), the success of which can be operator-dependent.

As aforesaid the panel of tumour marker antigens may be tailored having regard to the particular application. A panel of at least p53 and c-erbB2 is particularly useful for many types of cancer and can optionally be supplemented with other markers having a known association with the particular cancer, or a stage of the particular cancer, to be detected. For example for breast cancer the panel might include MUC 1 and /or c-myc and/or BRCA1 and/or BRCA2 and/or PSA whereas bladder cancer the panel might optionally include MUC 1 and/or c-myc, for colorectal cancer ras and/or APC , for prostate cancer PSA and/or BRCA 1 or for ovarian cancer BRCA1 and/or CA125. There are other preferred embodiments in which p53 or c-erbB2 are not necessarily essential. For example, in the case of breast cancer suitable panels could be selected from the following:

p53 and MUC 1 with optional c-erbB2 and/or c-myc, and/or BRCA1 and/or BRCA2 and/or PSA;

p53 and c-myc with optional c-erbB2 and/or MUC1 and/or BRCA1 and/or BRCA2 and/or PSA;

p53 and BRCA1 with optional c-erB2 and/or MUC 1 and/or c-myc and/or BRCA2 and/or PSA;

p53 and BRCA2 with optional c-erbB2 and/or MUC 1

and/or c-myc and/or BRCA1 and/or PSA;
c-erbB2 and MUC 1 with optional p53 and/or c-myc, and/or BRCA1 and/or BRCA2 and/or PSA;
c-erbB2 and c-myc with optional p53 and/or MUC1 and/or BRCA1 and/or BRCA2 and/or PSA;
c-erbB2 and BRCA1 with optional p53 and/or MUC 1 and/or c-myc and/or BRCA2 and/or PSA;
c-erbB2 and BRCA2 with optional p53 and/or MUC 1 and/or c-myc and/or BRCA1 and/or PSA;

In the case of colorectal cancer suitable panels could be selected from the following:
p53 and ras with optional c-erbB2 and/or APC;
p53 and APC with optional c-erbB2 and/or Ras;
Ras and APC with optional p53 and/or c-erbB2

In the case of prostate cancer suitable panels could be selected from the following:
p53 and PSA with optional BRCA1 and/or c-erbB2;
c-erbB2 and PSA with optional p53 and/or BRCA1.

In the case of ovarian cancer suitable panels could be selected from the following:
p53 and CA125 with optional c-erbB2 and/or BRCA1;
c-erbB2 and CA125 with optional p53 and/or BRCA1.

In a second aspect the invention provides a method of determining the immune response of a patient to two or more circulating tumour marker proteins or to tumour cells expressing said tumour marker proteins and identifying which one of said two or more tumour marker proteins elicits the strongest immune response in the patient, the method comprising contacting a sample of bodily fluids from said patient with a panel

of two or more distinct tumour marker antigens, measuring the amount of complexes formed by binding of each of said tumour marker antigens to autoantibodies present in the sample of bodily fluids, said autoantibodies being immunologically specific to said tumour marker proteins and using the measurement obtained as an indicator of the relative strength of the immune response to each tumour marker protein and thereby identifying which one of said two or more tumour marker proteins elicits the strongest immune response in the patient.

The assay described above, which may be hereinafter referred to as a 'selection assay' is useful in the selection of a course of vaccine treatment wherein the single tumour marker protein identified as eliciting the strongest immune response or a combination of markers eliciting strong immune response is/are used as the basis of an anti-cancer vaccine treatment.

Preferred tumour marker antigens for use in the selection assay are any of the tumour marker antigens mentioned above and preferably the antigens are labelled with biotin. The actual steps of detecting autoantibodies in a sample of bodily fluids may be performed in accordance with known immunological assay techniques, as described above for the panel assay.

The invention also provides methods for the detection or quantitative measurement of the immune response of a mammal to a circulating tumour marker protein or tumour cells expressing the tumour marker protein wherein the tumour marker protein is MUC1, c-erbB2, Ras, c-myc, BRCA1, BRCA2, PSA, APC, CA125 or p53, the method comprising the steps of contacting a sample of bodily fluids from the mammal with the

tumour marker antigen and determining the presence or absence of complexes of the tumour marker antigen bound to autoantibodies immunologically specific to the tumour marker protein or antigenic fragment thereof, whereby the presence of said complexes is indicative of the immune response to said circulating tumour marker protein or tumour cells expressing the tumour marker protein.

The assays described above, which may be hereinafter referred to as 'single marker assays', use a single type of tumour marker as antigen rather than using a panel of two or more tumour markers. The single marker assays may be used in any clinical situation, for example, screening for early neoplastic or carcinogenic change in asymptomatic patients, identification of individuals 'at risk' of developing cancer, early diagnosis and early detection of recurrence in a patient previously diagnosed as carrying tumour cells which patient has undergone treatment to reduce the number of said tumour cells or in predicting the response of a patient to a course of anti-cancer treatment, including surgery, radiotherapy, immune therapy, vaccination etc.

The single marker assays are particularly useful in situations where the tumour marker eliciting the strongest immune response in a given patient has been previously identified, possibly using the selection assay described above. For example, in a situation in which an initial selection assay has been performed to establish which tumour marker elicits the strongest immune response in a given patient, subsequent follow-up, detection of recurrence or monitoring of treatment may be carried out using a single marker assay to only detect or measure autoantibodies to that tumour marker

previously identified as eliciting a strong immune response in that patient.

The actual steps of detecting autoantibodies in a sample of bodily fluids may be performed in accordance with known immunological assay techniques, as described above for the panel assay. Preferably the tumour marker protein used as antigen is labelled with biotin so that it may be easily attached to a solid support by means of the biotin/avidin or biotin/streptavidin interaction.

In a further aspect, the present invention provides a preparation comprising a human MUC1 protein which MUC1 protein manifests all the antigenic characteristics of a MUC1 protein obtainable from the bodily fluids of a patient with advanced breast cancer.

Preferably the MUC1 protein exhibits altered affinity for the antibodies B55, C595, BC4W154, DF3, B27.29, 115D8, 27.1, SM3, Ma552, HMPV and BC2 compared to MUC1 protein isolated from normal human urine. Most preferably the MUC1 protein is isolated from the serum of one or more human patients with advanced breast cancer. This can be accomplished using the protocol given in the Examples listed herein.

As will be described in detail in Example 2, the present inventors have found immunological differences between MUC1 isolated from normal individuals and MUC1 isolated from patients with advanced breast cancer. Possibly as a result of these differences, the inventors have found that the MUC1 protein isolated from serum of patients with advanced breast cancer (hereinafter referred to as ABC MUC1) is more sensitive when used as antigen in an assay to detect autoantibodies specific to MUC1 than either MUC1

isolated from urine of normal individuals, synthetic MUC1 or MUC1 isolated from a range of different cultured cells. MUC1 isolated from the serum of patients with advanced breast cancer is therefore preferred for use as antigen in the panel assay method and the single marker assay methods described herein.

MUC1 has recently attracted interest as a target for immunotherapy of adenocarcinomas and several Phase I clinical trials involving different MUC1 vaccine substrates, adjuvants and carrier proteins have been carried out (Goydos, J. S. *et al.* (1996) *J Surgical Res.* **63**: 298-304; Xing, P. X. *et al.* (1995) *Int. J Oncol.* **6**: 1283-1289; Reddish, M. A. *et al.* (1996) *Cancer Immunol. Immunother.* **42**: 303-309; Graham, R. A. *et al.* (1996) *Cancer Immunol. Immunother.* **42**: 71-80). Methods for the detection of anti-MUC1 autoantibodies using MUC1 isolated from the serum of patients with advanced breast cancer as antigen will be of particular use in monitoring the success of MUC1 vaccine therapy. In this case the aim of the assay will be to detect anti-MUC1 antibodies produced in response to the vaccine rather than autoantibodies i.e. antibodies produced in response to an exogenous antigen introduced into the body by vaccination. Methods for the detection of autoantibodies directed to other tumour markers would also be of use in monitoring the success of vaccine therapy using the relevant tumour marker. For example, following vaccination with a p53 antigenic preparation, the presence of anti-p53 antibodies could be monitored using the assay based on the use of biotinylated p53 antigen described in the examples given below. Moreover, the panel assay method could also be used in monitoring the success of vaccine therapy, for

example, in a situation where an individual has been vaccinated with an antigenic preparation designed to elicit antibodies to two or more different tumour markers.

In a still further aspect the invention provides a method of detecting recurrent disease in a patient previously diagnosed as carrying tumour cells, which patient has undergone treatment to reduce the number of said tumour cells, which method comprises steps of contacting a sample of bodily fluids from the patient with MUC1 protein or an antigenic fragment thereof, determining the presence or absence of complexes of said MUC1 protein or antigenic fragment thereof bound to autoantibodies present in said sample of bodily fluids, said autoantibodies being immunologically specific to MUC1, whereby the presence of said complexes indicates the presence of recurrent disease in said patient.

The method described above may be repeated on a number of occasions to provide continued monitoring for recurrence of disease. The method is particularly preferred for the monitoring of patients previously diagnosed with primary breast cancer, colorectal cancer, prostate cancer or bladder cancer, which patients have undergone treatment (e.g. surgery) to remove or reduce the size of their tumour. In this instance, the presence of anti-MUC1 autoantibodies in the patient's serum after treatment may be indicative of recurrence of disease.

Also provided by the invention are assay kits suitable for performing the methods for the detection of autoantibodies described herein. Such kits include, at least, samples of the tumour marker antigens to be used as antigen in the assay and means for contacting

the sample to be tested with a sample of the antigen.

The contents of all documents, articles and references cited herein are incorporated herein by reference.

The present invention will be further understood with reference to the following Examples and the accompanying Figures in which:

Figure 1: shows the results of assays for autoantibodies to MUC1, p53 and c-erbB2 in samples of serum taken from 21 patients diagnosed with primary breast cancer. Panel A: anti-p53 autoantibodies; Panel B: anti-c-erbB2 autoantibodies and Panel C: anti-MUC1 autoantibodies. In each case, the dotted line represents the cut-off value for normality.

Figure 2: shows reactivity profiles of MUC1 protein isolated from normal human urine (panel A), ABC MUC1 isolated from the serum of patients with advanced breast cancer (panel B) or MUC1 isolated from the human breast cancer cell line ZR75-1 (panel C) with various monoclonal anti-MUC1 antibodies.

Figure 3: shows continuous monitoring for recurrent disease in three post-operative breast cancer patients. Quantitative assays for anti-MUC1, anti-c-erbB2 and anti-p53 autoantibodies and for the tumour marker CA15-3 (TM) were performed on samples of serum taken at two or three monthly intervals post-surgery.

Figure 4: shows the range of autoantibody levels found in assays for autoantibodies to c-erbB2, c-myc, MUC1 and p53 in normal individuals and patients with early primary breast cancer (PBC).

Figure 5: summarises the detection rate for primary breast cancer in an analysis of autoantibody levels in a series of healthy controls and patients with primary breast cancer, PBC subdivided by Stage 1-i.e. lymph node negative and Stage 2-i.e. lymph node positive and patients with metastatic cancer at 100% confidence.

Figure 6: summarises the detection rate for primary breast cancer in an analysis of autoantibody levels in a series of healthy controls and patients with PBC subdivided by Stage 1-i.e. lymph node negative and Stage 2-i.e. lymph node positive and patients with metastatic cancer at 95% confidence.

Figure 7: shows the sensitivity for primary breast cancer in an analysis of autoantibody levels in a series of healthy controls and patients with Stage 1 or Stage 2 primary breast cancer at 95% confidence.

Figure 8: shows the levels of autoantibodies to MUC1, p53 and c-erbB2 in the serum of three patients previously diagnosed with breast cancer measured sequentially during follow-up until the patient manifested recurrent disease.

Figure 9: shows the autoantibody levels in further samples from the second patient in Figure 10 (REC at 36 months) taken up to recurrence and during treatment for recurrence. Sequential measurements of established tumour markers reflecting tumour bulk (e.g. CA15-3 and CEA) were within the normal range throughout this period (data not shown).

Figure 10: shows follow-up autoantibody levels in

post-operative serum samples from two patients, one who did not develop recurrent disease (no REC) and the other who did (REC at 36 months).

Figure 11: summarises the detection rates in an analysis of autoantibody levels (p53, MUC1, c-erbB2 and c-myc) in samples of serum taken from patients with urologically benign disorders and various stages of bladder cancer.

* indicates patients which were benign with respect to urology (i.e. did not have a urological malignancy), but six cases (all with positive autoantibody status) had evidence of other malignancies.

** Other malignancies were:- lung cancer, skin cancer, adenocarcinoma of unknown primary. Evidence of other neoplasia consisted of:- pleural effusion, ovarian cysts, colon polyps.

Figure 12: summarises the detection rate for colorectal cancer in an analysis of autoantibody levels in the serum of healthy controls, patients with colonic polyps and patients with colorectal cancer at 100% confidence compared to a pre-defined group of healthy controls.

Figure 13: summarises the detection rate for colorectal cancer in an analysis of autoantibody levels serum of healthy controls, patients with colonic polyps and patients with colorectal cancer at at 95% confidence compared to a pre-defined group of healthy controls.

Figure 14: summarises the detection rate in an analysis of autoantibody levels in the serum of

healthy controls, patients with primary breast cancer and asymptomatic women known to be BRCA1 mutant carriers at 100% confidence compared to a pre-defined group of healthy controls.

Figure 15: summarises the detection rate for prostate cancer in an analysis of autoantibody levels in the serum of healthy controls and patients with prostate cancer at 95% confidence compared to a pre-defined group of healthy controls.

Examples

Example 1-Isolation of ABC MUC1 from advanced breast cancer patients.

Method

ABC MUC1 was purified from pooled sera taken from 20 patients with advanced breast cancer using immunoaffinity chromatography as follows:

The mouse monoclonal anti-MUC1 antibody B55 (also known as NCRC 11 and described by Ellis *et al.* (1984) *Histopathology.* 8: 501-516 and in International patent application No. WO 89/01153) was conjugated to CNBr-sepharose beads. Pooled sera from patients diagnosed with advanced breast cancer was diluted 1/10 in phosphate buffered saline (PBS) and then incubated with the antibody conjugated sepharose beads (25ml diluted sera to 1ml packed volume of beads) overnight at 4°C with rolling. The beads were then packed by centrifugation and the supernatant removed. In order to wash away unbound serum components the beads were resuspended in PBS, rolled for 10 minutes, packed by

centrifugation and the supernatant removed. This washing sequence was repeated 5 times (or until A280nm of the supernatant was ~0). The washed beads were then resuspended in 0.25M glycine pH 2.5, rolled at room temperature for 10 minutes, packed by centrifugation and the supernatant removed. This supernatant was adjusted to pH 7 by the addition of Tris and stored at 4°C labelled 'glycine fraction'. The beads were then resuspended in 1ml 25mM diethylamine (DEA) pH11, rolled at room temperature for 10 minutes, packed by centrifugation and the supernatant removed. This supernatant was again adjusted to pH 7 by the addition of Tris and stored at 4°C labelled '25 DEA fraction'. The beads were finally resuspended in 1ml 100mM DEA pH11, rolled at room temperature for 10 minutes, packed by centrifugation and the supernatant removed. The final supernatant was again adjusted to pH 7 by the addition of Tris and stored at 4°C labelled '100 DEA fraction'. The MUC1 content of the three fractions (glycine fraction, 25 DEA fraction and 100 DEA fraction) was confirmed by ELISA using the mouse monoclonal anti-MUC1 antibody C595 (commercially available from Serotec).

Example 2-Immunological characterisation of ABC MUC1 isolated from the serum of patients with advanced breast cancer.

ABC MUC1 isolated from the serum at least 20 patients with advanced breast cancer according to the procedure described in Example 1 can be distinguished from MUC1 isolated from the urine of normal human subjects (normal human urinary MUC1) on the basis of altered affinity for the following mouse monoclonal anti-MUC1 antibodies:

B55 (NCRC 11)	
C595	
BC4W154	Obtainable from Hybritech, Inc
DF3	Obtainable from Centocor
B27.29	Obtainable from Biomira, Inc
115D8	Obtainable from Centocor
27.1	Obtainable from Austin Research Institute
SM3	Obtainable from the Imperial Cancer Research Fund
Ma552	Obtainable from CanAg
HMPV	Obtainable from Austin Research Institute
BC2	Obtainable from Austin Research Institute

Normal urinary MUC1 is available from Dr M.R. Price, Cancer Research Laboratories, The University of Nottingham, University Park, Nottingham. NG7 2RD, United Kingdom.

The affinity of each of the above antibodies for ABC MUC1, normal human urinary MUC1 and also MUC1 protein purified from the human breast cancer cell line ZR75-1 (purified from a tissue culture supernatant by gel filtration) was measured by performing colorimetric ELISA assays using each of the different antibodies and secondary anti-immunoglobulin antibodies conjugated to HRP. Following addition of the colorimetric substrate (TMB), measurements were taken of OD at 650nm. The results of the ELISA assays are presented graphically in Figure 2. Values of Kd for the binding of several of these antibodies to ABC MUC1 and normal human urinary MUC1 are summarised in Table 1:

Table 1: Kd values for binding of monoclonal antibodies to ABC MUC1 and normal human urinary MUC1.

Monoclonal	Kd vs ABC MUC1	Kd vs urinary MUC1
BC4W154	$2.4x10^{-7}$	$1.7x10^{-9}$
115D8	$1x10^{-8}$	$3.38x10^{-8}$
C595	$2.4x10^{-8}$	$2.5x10^{-8}$

Example 3-Cloning of biotinylated p53.

Method

Commercially available cDNA for p53 (*E. coli* clone pBH53, deposited in the American Type Culture Collection under accession number 79110) was cloned into the PinPoint™ plasmid vector (Promega Corporation, Madison WI, USA) using standard molecular biology techniques. The PinPoint™ vector is designed to facilitate the production of fusion proteins comprising a biotinylation domain (consisting of a fragment of a biotin carboxylase carrier protein) fused N-terminally to the target protein of interest. Care was therefore taken during the cloning procedure to ensure that the reading frame of p53 was maintained in the fusion protein. Procedures for cloning in PinPoint™ vectors are described in detail in the Promega Protocols and Applications Guide obtainable from Promega Corporation, Madison WI, USA.

Fusion proteins expressed from the PinPoint ™ vector in *E. coli* are biotinylated by an enzyme system of the *E. coli* host cells and may therefore be purified or bound to an assay plate using conventional avidin or streptavidin technology. For example, procedures for purification of the fusion protein using avidin covalently attached to a polymethacrylate resin are described in the Promega Protocols and

Applications Guide obtainable from Promega Corporation, Madison WI, USA.

Example 4-Cloning of c-erbB2.

Method

Full-length cDNA encoding c-erbB2 was cloned from the human breast cancer cell line ZR75-1, which can be induced to up-regulate c-erbB2 expression by treatment with the anti-cancer drug tamoxifen.

Two T25 flasks of sub-confluent ZR75-1 cells (avaiable from the American Type Culture Collection and from the European Collection of Cell Cultures, deposit number ATCC CRL1500) grown in RPMI plus 10% foetal calf serum were induced to express c-erbB2 by 4 day stimulation with tamoxifen at 7.5μM (see Warri *et al.* (1996) *Eur. J. Cancer.* **32A**: 134-140). The cells were then harvested using trypsin/EDTA and washed three times with PBS.

mRNA was extracted from the cell pellet using a Dynabead mRNA purification kit according to the manufacturer's recommended protocol. The mRNA was then used as a template for first strand cDNA synthesis using the Pharmacia Ready-to-go™ T primed first strand cDNA synthesis kit. cDNA/mRNA was then blunt end ligated into the EcoRV site of the PinPoint™ vector. The ligation products were then transformed into Top 10 F *E. coli* cells (Invitrogen) following the manufacturer's supplied protocol and the transformed cells grown overnight on LB agar plates containing ampicillin. Colonies of the transformed *E. coli* were copied onto nitrocellulose filter and then grown for 2 hours on LB agar containing ampicillin and IPTG (1mM). The colonies on the nitrocellulose filter were fixed and lysed (15 minutes in the presence of chloroform

vapour followed by 18 hours in 100mM Tris/HCL pH 7.8; 150mM NaCl; 5mM MgC12; 1.5% BSA; 1µg/ml DNase 1; 40µg/ml lysozyme).

Screening for colonies expressing anti-c-erbB2 reactive protein was carried out as follows:

1. Wash nitrocellulose filter three times in TNT (10mM Tris/HCl pH 8; 150mM NaCl; 0.05% Tween 20) then block for 60 minutes in TNT + 5% dried milk protein.

2. Incubate nitrocellulose filter for 2 hours at room temperature with mouse anti-c-erbB2 antibody (Ab-3 from Oncogene Research Products, Calbiochem).

3. Wash the filter three times in TNT then incubate overnight at 4°C with anti-mouse HRP conjugate.

4. Wash filter three times in TNT, twice in TN (10mM Tris/HCl pH 8; 150mM NaCl) then visualise colonies expressing anti-c-erbB2 reactive protein using chloronaphthol (6mg chloronaphthol in TN + 6µl 30% H_2O_2).

5. After development (approximately 20 minutes treatment with chloronaphthol as described in step 4) wash filter with water and allow to air dry.

Colonies identified as positive for c-erbB2 expression were picked and grown up overnight in liquid culture of LB + ampicillin and small amounts of plasmid DNA and protein were prepared from the culture

for analysis. Plasmids containing a c-erbB2 cDNA insert were identified using restriction enzyme digestion and PCR using a primer pair specific to the published c-erbB2 cDNA sequence, described by Yazici, H. *et al.*(1996) *Cancer Lett.* 107: 235-239. DNA sequence analysis could then be used to confirm 1) the presence of a c-erbB2 insert and 2) that the reading frame of c-erbB2 is maintained in the resultant biotinylated fusion protein. Protein samples prepared from *E. coli* cultures carrying a plasmid with a c-erbB2 insert were analysed by SDS-PAGE and western blotting to ensure that the correct protein was being expressed.

Example 5-Detection of the immune response of patients with primary breast cancer using a panel assay.

Methods:

(A) Preparation of biotinylated antigen

E. coli transformed with the appropriate PinPoint™ plasmid expressing biotinylated antigen were grown in a 5ml overnight culture (LB + amp + biotin) and the overnight culture used to inoculate a 150ml culture. The 150ml culture was grown to OD 0.4-0.6 then expression of the fusion protein was induced by the addition of IPTG to a final concentration of 1mM and the induced culture incubated at 25°C. The bacterial cells were harvested by centrifugation and then lysed by gentle sonication in a Tris/EDTA buffer containing the protease inhibitor PMSF. Cellular debris was removed by centrifugation at ~50,000g and the resultant particle-free supernatant assayed by avidin ELISA to confirm the presence of biotinylated

protein.

(B) c-erbB2/p53 autoantibody assay method

1. Standard 96 well microtitre assay plates were coated with avidin, using 50µl of a 1µg/ml solution per well, and allowed to air dry overnight. The plates were then washed once with PBS/Tween to remove residual salt crystals, blocked for 60 minutes with a solution of 2% (w/v) PVP (polyvinylpyrolidone 360) in PBS and washed three times using PBS/Tween.

2. Particle free supernatant containing the appropriate biotinylated antigen (prepared as described in section (1) above) was plated out at 50µl per avidin-coated well and then incubated for 60 minutes at room temperature with shaking to allow the biotin/avidin binding reaction to take place. The plates were then washed four times with PBS/Tween.

3. Serum samples to be tested for the presence of autoantibodies (diluted 1/50 and 1/100 in PBS)were plated out in triplicate (50µl per well) and then incubated for 60 minutes with shaking to allow formation of any autoantibody/antigen complexes. Plates were then washed four times with PBS/Tween to remove unbound serum components.

4. 50µl of HRP conjugated anti-human IgG/IgM antibody (obtained from Dako and used at a dilution recommended by the manufacturer) was added to each well and incubated for 60 minutes

at room temperature with shaking. The plates were then washed again four times with PBS/Tween.

5. 50µl of TMB was added to each well and measurements of OD at 650nm for each well of the assay plate were taken kinetically over a period of 10 minutes.

For each antigen, control assays were performed following the procedure described above but using a sample of protein induced from *E. coli* transformed with a control PinPoint™ vector containing an out-of-frame cDNA instead of the particle free supernatant containing biotinylated antigen. As it will be apparent to persons skilled in the art, the above methodology can be adapted for use in the detection of autoantibodies of any specificity with use of an appropriate biotinylated antigen.

(C) MUC1 autoantibody assay

1. ABC MUC1 isolated from the serum of patients with advanced breast cancer according to the method of Example 1 (all three fractions pooled) was diluted appropriately in PBS, plated out on a 96 well microtitre assay plate at 50µl per well and left to dry overnight. The plate was then washed once with PBS/Tween to remove residual salt crystals, blocked for 60 minutes using a solution of 2% (w/v) PVP in PBS and washed three times with PBS/Tween.

2. Serum samples to be tested for the presence of autoantibodies (diluted 1/50 and 1/100 in PBS) were plated out in triplicate, adding 50µl per

well, and incubated for 60 minutes at room temperature with shaking. The plate was then washed four times with PBS/Tween.

3. 50µl of HRP conjugated anti-human IgG/IgM antibody (obtained from Dako and used at a dilution recommended by the manufacturer) was added to each well and incubated for 60 minutes at room temperature with shaking. The plates were then washed again four times with PBS/Tween.

4. 50µl of TMB was added to each well and measurements of OD at 650nm for each well of the assay plate were taken kinetically over a period of 10 minutes.

Results

Pre-operative blood samples taken from 21 patients diagnosed with primary breast cancer were assayed for the presence of autoantibodies against MUC1, p53 and c-erbB2. The results of these assays are shown in Figure 1 and summarised in Table 2, on page 30.

Table 2

Sample	anti-p53	Prediction	anti-c-erbB2	Prediction	anti-MUC1	Prediction	Combined
1	+	cancer	-	normal	+	cancer	CANCER
2	+/-	?	+/-	?	+/-	?	cancer
3	+	cancer	+/-	?	+	cancer	CANCER
4	+	cancer	+	cancer	+	cancer	CANCER
5	+	cancer	+	cancer	+/-	?	CANCER
6	-	normal	+	cancer	+/-	?	cancer
7	+	cancer	+	cancer	+	cancer	CANCER
8	+/-	?	+	cancer	+/-	?	CANCER
9	+	cancer	+	cancer	+	cancer	CANCER
10	+	cancer	+	cancer	-	normal	CANCER
11	+/-	?	+	cancer	+	cancer	CANCER
12	-	normal	+	cancer	-	normal	cancer
13	+	cancer	-	normal	+	cancer	CANCER
14	+/-	?	+	cancer	+	cancer	CANCER
15	+	cancer	-	normal	+	cancer	CANCER
16	-	normal	-	normal	+/-	?	?
17	+/-	?	-	normal	+	cancer	cancer
18	+	cancer	+	cancer	+	cancer	CANCER
19	+	cancer	+	cancer	+	cancer	CANCER
20	+	cancer	-	normal	+	cancer	CANCER
21	+	cancer	+/-	?	-	normal	cancer

Figure 1 shows the results of the assays for autoantibodies specific to MUC1, c-erbB2 and p53. For each set of data the dotted line represents the cut-off value for normality. For the purposes of this study the normal control patients were women who clinically and/or mammographically had no evidence of breast cancer at the time of taking the serum sample. In order to establish the cut-off value for normality, control assays were performed on a total of 30 normal patients. Values below the dotted line fall within the normal control range and were scored as negative (-) in Table 2 whereas values above the dotted line were scored as positive (+). Values which were difficult to score as negative or positive with a reasonable degree of certainty were scored +/-. Patients scoring positive in at least two of the assays were identified as strongly positive for breast cancer (indicated "CANCER" in Table 2) ; patients scoring positive in at least one of the assays were identified as probable for breast cancer (indicated "cancer" in Table 2).

The results presented illustrate the predictive value of the three autoantibody assays both when used individually and when used as a panel. The use of a single assay to predict breast cancer gave approximately 40% of the results as a false negatives. However, by combining the results from all three assays only one patient appeared as a false negative (<5%), 71% of patients were scored as strongly positive for breast cancer (i.e. positive in at least two assays) and 23% of patients were scored as probable for breast cancer (i.e. positive in at least one assay). The results also show that a group of patients which have all been diagnosed with primary breast cancer have different serological profiles in

terms of the immune response to their cancer. Thus, no single one of the three autoantibody assays would be useful in all primary breast cancer patients.

Example 6-Cloning of a ras antigen.

Method

cDNA encoding a mutant oncogenic form of ras (designated K-*ras*) was cloned from the cell line KNRK (Rat kidney, Kirsten MSV transformed, see Aaronson, S.A. and Weaver, C.A. (1971) J. Gen. Virol. 13: 245-252; ATCC accession number CRL 1569). mRNA was extracted from the cell pellet using a Dynabead mRNA purification kit according to the manufacturer's recommended protocol. cDNA synthesis, cloning into the EcoRV site of the PinPoint™ vector and transformation of *E. coli* was carried out as described in Example 4. Clones expressing ras were then identified by expression screening using the anti-ras antibody F234-4.2 from Calbiochem.

Example 7 Cloning of c-myc.

Method

cDNA encoding human c-myc was cloned from the breast cancer cell line T47-D (European Collection of Animal Cell Cultures accession number 85102201). mRNA was extracted from the cell pellet using a Dynabead mRNA purification kit according to the manufacturer's recommended protocol. cDNA synthesis, cloning into the EcoRV site of the PinPoint™ vector and transformation of *E. coli* was carried out as described in Example 4. Clones expressing c-myc were then identified by expression screening using the anti-c-myc antibody 4111.1 from Unilever.

Example 8 Assay for ras and c-myc autoantibodies.

Biotinylated c-myc and ras antigens were prepared from *E. coli* transformed with the appropriate PinPoint™ plasmid vector expressing biotinylated c-myc or biotinylated ras, as described in Example (5), part (A). The assays for c-myc and ras autoantibodies were then performed according to the protocol described in Example (5), part (B).

Example 9 Method of detecting recurrent disease in a patient previously diagnosed as carrying tumour cells.

A group of nine patients previously diagnosed with primary breast cancer were selected. Pre-operative serum samples were taken from each of these patients prior to surgery for the removal of the primary breast cancer. Follow-up serum samples were then taken postoperatively at 2 or 3 monthly intervals and during the same period of time the patients were assessed clinically for signs of recurrent disease. None of the patients received any post-operative therapy until recurrence was diagnosed clinically. The preoperative and post-operative serum samples from each of the patients were assayed for the presence of autoantibodies to MUC1, c-erbB2 and p53, using the assay methods described above under Example 5, and also for the presence of the commonly used serum tumour marker protein CA15-3. The results of these assays are summarised in Table 3, on pages 35 and 36 and results for three of the nine patients are presented graphically in Figure 3.
Clinical signs of recurrent disease were scored as follows:

LN recurrent disease in the lymph nodes

LR local recurrence

METS distant metastases present

Results

In each of the patients at least one class of autoantibody was observed to remain above normal level. This suggests continued presence of the tumour marker (immunogen) and hence continued presence of tumour. Serum levels of the tumour marker protein CA15-3 were not found to be predictive of recurrent disease.

Table 3

Patient	Sample date	CA 15-3	Anti-p53	Prediction	Anti c-erbB2	Prediction	Anti MUC1	Prediction	Predicted	Recurrence	Date of first recurrence	DFI (months)
0001	12/88	11	–		+	Cancer	+	Cancer	CANCER	–		
	03/87	12	–		+	Cancer	+	Cancer	CANCER	–		
	05/87	13	–		+	Cancer	+	Cancer	CANCER	–		
	08/87	22	+/–	?	+	Cancer	+	Cancer	CANCER	–		
	11/87	56	+/–	?	+	Cancer	+	Cancer	CANCER	METS		
	12/87	79	+/–	?	+	Cancer	+	Cancer	CANCER	METS		11
0002	01/87	16	–		+	Cancer	+/–	?	Cancer	–		
	05/87	8	–		+	Cancer	+/–	?	Cancer	–		
	08/87	10	–		+	Cancer	+	Cancer	CANCER	–		
	11/87	12	+/–	?	+	Cancer	+	Cancer	CANCER	–		
	02/88	16	–		+	Cancer	+	Cancer	CANCER	–	February 1989	23
0003	02/87	10	–		+	Cancer	–		Cancer	–		
	05/87	7	+	Cancer	+	Cancer	–		CANCER	–		
	08/87	8	+	Cancer	+	Cancer	–		CANCER	–		
	11/87	12	+	Cancer	+	Cancer	–		CANCER	–		
	02/88	12	+	Cancer	+	Cancer	–		CANCER	–		
	05/88	11	–		+	Cancer	–		Cancer	–	December 1989	34
0004	02/87	8	+	Cancer	++	Cancer	–		CANCER	–		
	04/87		+	Cancer	+	Cancer	–		CANCER	–		
	06/87	4	+	Cancer	+	Cancer	–		CANCER	–		
	12/87	0.4	+	Cancer	++	Cancer	–		CANCER	–		
	03/88	7	++	Cancer	++	Cancer	–		CANCER	–	February 1993	71

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Table 3 continued

Patient	Sample date	CA 15-3	Anti-p53	Prediction	Anti c-erbB2	Prediction	Anti MUC1	Prediction	Predicted	Recurrence	Date of first recurrence	DFI (months)
0005	03/87	16	+/–	?	+	Cancer	–		Cancer	–		
	06/87	13	+/–	?	+	Cancer	–		Cancer	–		
	09/87	14	+	Cancer	+	Cancer	+/–	?	CANCER	–		
	12/87	17	+/–	?	+	Cancer	+/–	?	CANCER	–		
	03/88	16								–		
	05/88									LN		15
0006	05/87	12	–		+	Cancer	+	Cancer	CANCER	–		
	07/87	15	–		+	Cancer	+	Cancer	CANCER	–		
	09/87	9	+/–	?	+	Cancer	+/–	?	Cancer	LR		4
	11/87	12	–		+	Cancer	+/–	?	Cancer	–		
	03/88	15	–		+/–	?	–			–		
	05/88	13	–		+/–	?	–			–	November 1988	
000&	06/87	26	+	Cancer	++	Cancer	–		CANCER	–		
	08/87	28	+	Cancer	+	Cancer	–		CANCER	–		
	10/87	42	+	Cancer	+	Cancer	–		CANCER	–		
	12/87	105	+	Cancer	++	Cancer	+	Cancer	CANCER	METS	December 1987	6
0008	06/87	48	+	Cancer	+	Cancer	+	Cancer	CANCER	–		
	08/87	30	+	Cancer	+	Cancer	+	Cancer	CANCER	–		
	10/87	17	+	Cancer	+	Cancer	+	Cancer	CANCER	–		
	01/88	14	+	Cancer	+	Cancer	+	Cancer	CANCER	–		
	05/88	22	+	Cancer	+	Cancer	+/–	?	CANCER	LR	May 1988	11
0009	05/87	17	–		+/–	?	–			–		
	08/87	17	–		+	Cancer	–		Cancer	–		
	11/87	18	–		+	Cancer	–		Cancer	LR		6
	01/88	31	–		+	Cancer	+/–	?	Cancer	METS		8

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Example 10 Retrospective analysis of a well characterised series of healthy controls and patients with early breast cancer.

The above-described methods for detecting autoantibodies to MUC1, p53, c-erbB2 and c-myc were used to carry out a retrospective study on a large number of early (stage 1 and 2) breast cancer sera as well as a large number of control serum samples from individuals with no evidence of malignancy (control group). The serum samples from patients were all taken within a 4 week pre-operative period. At the same time, the serum samples were assayed for the presence of circulating antigen (MUC1 and c-erbB2) using conventional tumour marker kits (used normally in advanced disease only). This would allow an assessment of whether the autoantibody assays are more sensitive than the conventional antigen assays. As used herein, the terms early or primary breast cancer means that the primary tumour has a diameter of less than 5cm. Stage 1 early breast cancer is defined as lymph node negative; Stage 2 early breast cancer is defined as lymph node positive.

In total, pre-operative serum samples from 200 patients diagnosed with primary breast cancer and 100 normal control samples were assayed for autoantibodies against MUC1, p53, c-erbB2 and c-myc. The results are summarised in Tables 4-7 and Figures 4-7.

Figure 4 depicts the range of autoantibody levels found for each assay in normal individuals and patients with early breast cancer. It is apparent that cancer patients have a considerably higher level of circulating autoantibodies to these markers than do normal individuals. Using the range for the normal individuals it is possible to set a 'cut-off' above

which no normal values should lie. Therefore, samples with autoantibody levels above this cut-off can be deemed to be positive for cancer. Cut-off points determined in this manner were used to score the results of the retrospective study in early breast cancer patients.

The results presented in Tables 4-7 and Figures 5-7 demonstrate the predictive value of the four autoantibody assays both individually and when used in combination as a panel of assays. Table 4 indicates the increased sensitivity of combining the results of a number of assays. By using one assay on its own, less than 50% of cancers are detected, however the power of detection increases as more assays are added to the panel until the combination of all four assays allows 82% of primary cancers to be detected. Figure 7 shows the percentage of samples which are positive in 0 out of 4 assays up to 4 out of 4 assays. This provides good evidence that the panel assay is more powerful in the detection of cancer than any one single marker assay since not all patients with cancer have raised autoantibodies to all markers.

Tables 5-7 summarise the detection rates in stage 1, stage 2 and in early breast cancer (i.e. stage 1 and 2) for various combinations of autoantibody assays. The use of a single autoantibody assay to predict breast cancer gives approximately 60-70% of the results as false negatives in the stage 1 group; and 50-60% in stage 2. However, by combining the results from all four assays, 76% of stage 1 and 89% of stage 2 cancers were positive in one or more assay. The overall detection rate for early breast cancer (i.e. both stage 1 and stage 2 cancers) using this system was 82%. In both stage 1 and stage 2 cancer,

assaying for autoantibodies to MUC1 appeared to add predictive power to any combination of assays.

The results for this study were obtained using a 100% confidence limit, in other words for a result to be deemed positive it had to fall above the cut-off for readings in the normal range. This normal range was previously evaluated from a large number of normal individuals and then confirmed using the control group of 100 normal individuals mentioned above. Therefore, within the normal control group, none of the samples were found to be positive, meaning that the sensitivity of the panel of autoantibody assays was 100% for the detection of early breast cancer (Figure 5).

Figures 6 and 7 demonstrate the detection rates which are achievable if specificity is reduced from a 100% confidence level (no false positives) to a 95% confidence level, where some degree of false positive detection is expected. In this case, the cut-off point is defined as the mean value plus twice the standard deviation of the normal sample range. Using this cut-off point, approximately 5% of the normal samples were determined to be positive for cancer (i.e. false positives); whilst detection of primary cancer increased to approximately 94% (i.e. 6% false negatives). Again, the greatest percentage of the sample group were positive in only 1 out of the 4 assays, however, the percentage of samples that were positive in all 4 assays increased considerably.

Since the above study was carried out retrospectively, clinical data was available regarding the initial diagnosis as well as clinical data regarding the post-operative outcome (i.e. follow-up data). This allowed analysis of the prognostic value

of the data obtained from the autoantibody assays. Table 8 shows the correlations between serum levels of autoantibodies to MUC1, p53, c-erbB2 and c-myc and a number of clinical factors. For instance, the presence of autoantibodies to any of the 4 tumour associated proteins (MUC1, p53, c-erbB2 or c-myc) appears to correlate with the development of a recurrence. In other words, those patients who had autoantibodies were more likely to go on to develop a recurrence of their disease. In the case of autoantibodies to MUC1, c-myc and c-erbB2, this was most likely to be distant metastases, only autoantibodies to p53 were not associated with the later development of distant metastases with any statistical significance. In fact, the presence of autoantibodies to p53 was the weakest indicator of a later recurrence of disease; furthermore, p53 autoantibodies correlated with disease free interval.

Table 9 presents an analysis of whether the degree of autoantibody positivity may be of value in the prediction of which stage 1 tumour will go on to develop a recurrence. At the present time, there is little to indicate at the time of diagnosis whether a patient with a stage 1 tumour (i.e. no evidence of spread of tumour to the lymphatic system) will go on to develop recurrent disease. As can be seen in Table 9, of those patients with stage 1 tumours from the sample group that went on to develop recurrent disease, 71% were positive in two or more autoantibody assays. Of the patients with stage 1 tumours that have not yet recurred, only 30% were positive in two or more autoantibody assays.

Table 4: Sensitivity of autoantibody assays in the detection of early breast cancer.

	% PBC positive
Single marker assay	35-47
Two marker assay	51-60
Three marker assay	63-76
Four marker assay	82

Table 5: Sensitivity of autoantibody panel assays in the detection of stage 1 breast cancer.

	p53	c-erbB2	c-myc	MUC1
p53	38	48	58	59
c-erbB2		31	50	51
c-myc			41	55
MUC1				38
p53/c-erbB2			61	66
p53/c-myc				73
c-erbB2/c-myc				65
p53/c-erbB2/c-myc				76

Table 6: Sensitivity of autoantibody panel assays in the detection of stage 2 breast cancer.

	p53	c-erbB2	c-myc	MUC1
p53	40	56	55	73
c-erbB2		42	56	73
c-myc			33	69
MUC1				56
p53/c-erbB2			65	84
p53/c-myc				80
c-erbB2/c-myc				84
p53/c-erbB2/c-myc				89

Table 7: Sensitivity of autoantibody panel assays in the detection of primary breast cancer.

	p53	c-erbB2	c-myc	MUC1
p53	38	51	57	64
c-erbB2		35	53	59
c-myc			37	60
MUC1				47
p53/c-erbB2			63	73
p53/c-myc				76
c-erbB2/c-myc				72
p53/c-erbB2/c-myc				82

Table 8: Correlations between serum autoantibody level and various clinical factors.

FACTOR	MUC1	p53	c-erbB2	c-myc
recurrence	✓	1✓4	✓	✓
local recurrence	1✓2	1✓2	1✓2	1✓4
distant metastases	✓	×	✓	✓
stage	×	×	×	×
grade	×	×	×	×
family history	×	×	×	×
disease free interval	×	✓	×	×
age	×	×	×	×
menopausal status	×	×	×	×

Key:

✓ Good correlation

1✓2 Moderate correlation

1✓4 Weak correlation

× No correlation

Table 9: Analysis of the degree of positivity in autoantibody assays for recurrent and non-recurrent stage 1 breast cancer tumours.

	Negative-no autoantibodies detected	+ve auto-antibodies to one marker	+ve auto-antibodies to 2-4 markers
Recurrent	12%	17%	71%
Non-recurrent	22%	48%	30%

Example 11 Detection of autoantibodies in sequential serum samples-application to the monitoring of disease progression.

This study was carried out in order to assess whether autoantibody assays could be useful in the

earlier detection of recurrent disease.

Levels of autoantibodies to MUC1, p53 and c-erbB2 in the serum of patients previously diagnosed with breast cancer were measured sequentially during follow-up until the patient manifested recurrent disease. The results are summarised in Figures 8-10. All three patients went on to develop recurrent disease. In all three patients, autoantibody levels were indicative of the presence of cancer. However, there is no evidence from this group that autoantibody levels decrease after removal of the primary tumour. Figure 10 shows the levels of autoantibodies post-operatively of a patient with non-recurrent disease and a patient with recurrent disease. Autoantibody levels in the patient with non-recurrent disease remained below the cut-off point during the period of sample collection (48 months). In the second patient, whose disease recurred at 36 months, autoantibody levels are seen to be steadily rising towards the cut-off point, with c-erbB2 autoantibodies rising above cut-off. Furthermore, as can be seen in Figure 9, when further sequential samples are added to the analysis, 3 out of the 4 assays become positive for cancer and these levels then decrease again once treatment of the recurrence is underway. This data supports the utility of autoantibody assays in the earlier detection of recurrent disease.

Example 12 Analysis of a series of patients with bladder cancer and benign urological disorders.

Serum samples were collected from a group of 80 patients with bladder cancer/benign urological disorders and analysed for the presence of autoantibodies to MUC1, p53, c-erbB2 and c-myc using

the assay methods described above.

The data summarised in Table 10 shows that single assay sensitivities for bladder cancer detection range from 15-50% (as opposed to 35-47% for breast cancer). The detection sensitivity using all 4 assays was 80%, similar to that found for early breast cancer.

Figure 11 shows the break down of detection rates between urologically benign disorders ('benign') and the three stages of bladder cancer. Upon further investigation of the relevant clinical data it became apparent that 6 of the patients in the 'benign' group had evidence of other malignancies. These other malignancies were lung cancer, skin cancer and adenocarcinoma. Evidence of other malignancies were: pleural effusion, ovarian cysts and colon polyps. Serum samples from all 6 of these patients had been scored as positive for cancer using the panel of autoantibody assays, illustrating the general application of the panel assay to the detection of cancers. Furthermore, it is known that some patients with stage PT1/2 and PT3/4 disease had previously received systemic therapy.

Table 10: Sensitivity of autoantibody assays in the detection of bladder cancer.

	% positive
Single marker assay	15-50
Two marker assay	28-73
Three marker assay	46-76
Four marker assay	80

Table 11: Sensitivity of autoantibody panel assays in the detection of bladder cancer.

	p53	c-erbB2	c-myc	MUC1
p53	50	73	73	73
c-erbB2		17	28	36
c-myc			15	35
MUC1				24
p53/c-erbB2			76	76
p53/c-myc				75
c-erbB2/c-myc				46
p53/c-erbB2/c-myc				80

Example 13

Sensitivity of autoantibody assay in diagnosis of colorectal cancer.

An autoantibody assay as previously described was carried out on serum samples from patients with colorectal cancer using the tumour antigens c-myc, p53, c-erbB2 and K-ras individually and as a panel. The results are shown in figures 12 and 13. As has been demonstrated previously increased sensitivity is shown when a panel of antigens is used.

Example 14

Use of BRCA1 in panel assay for detection of breast cancer.

A BRCA1 antigen suitable for use in the detection of anti-BRCA1 autoantibodies was cloned from the breast cancer cell line MCF7 using an RT-PCR strategy. Briefly, mRNA isolated from MCF7 cells was reverse transcribed to give first-strand cDNA. These cDNA was used as a template for PCR using a primer pair

designed to amplify a product covering the first 1500 base pairs of the BRCA1 cDNA but including a known mis-match mutation that leads to an early stop codon and therefore the production of truncated protein. Different sites for restriction enzyme digestion were also incorporated into the forward and reverse PCR primers to facilitate the cloning of the PCR product. The PCR primers were as follows:

5'-GAC AGG ATC CGG ATG GAT TTA TCT GCT CTT CGC GTT G

5'-GCG GCC GCC CTC ATG TAG GTC TCC TTT TAC GC

The PCR product obtained using these primers was then cloned into the PinPoint™ vector and used to transform *E. coli* Top 10 F cells, as described hereinbefore. Clones expressing the fusion protein of truncated BRCA1 antigen fused in-frame to the N-terminal biotinylation domain were then identified by expression screening, according to the procedure described in Example 4, using the antibody MAB4132 from Chemicon.

Biotinylated truncated BRCA1 antigen is then prepared from *E. coli* transformed with the appropriate PinPoint™ plasmid vector expressing the fusion protein, as described in Example (5), part (A). The assay for BRCA1 autoantibodies is then performed according to the protocol described in Example (5), part (B).

Figure 14 shows the results of a study in which the above-described assays for autoantibodies to c-myc, p53, c-erbB2, MUC1 and BRCA1 were performed individually, as a panel and as a panel without BRCA1 to detect autoantibodies in samples of serum taken

from normal individuals, patients diagnosed with primary breast cancer and BRCA1 mutation carriers. As demonstrated previously, increased sensitivity is shown when a panel of markers is used.

Example 15

Use of autoantibody panel assay for detecting prostate cancer, incorporating PSA.

cDNA encoding human PSA was cloned from the cell line T47-D using a protocol similar to that described above for the cloning of c-erbB2. Briefly, the T47-D cells were first stimulated with Apigenin at 10-5M as described by Rosenberg *et al.* (1998) *Biochem Biophys Res Commun.* **248**: 935-939. mRNA was then extracted and cDNA synthesis, ligation into PinPoint™ and transformation of *E. coli*. performed as described in Example 4. Clones expressing PSA were identified using an anti-PSA antibody. Biotinylated PSA antigen was prepared from *E. coli* transformed with the PinPoint™ vector expressing biotinylated PSA according to the protocol described in Example (5), part (A). The assay for PSA autoantibodies was then performed according to the protocol described in Example (5), part (B).

An autoantibody assay using the methods described above was carried out on patients with prostate cancer using c-myc, p53, c-erbB2, PSA and MUC 1 individually and as a panel. The results are shown in figure 15 and confirm the increased sensitivity of such a panel for detection of prostate cancer.

Example 15

Other tumour marker antigens.

CA125 can be affinity purified from the ovarian

cancer cell line OVRCAR-3 (available from the ATCC) using Mab VK-8, as described by Lloyd, K. O. *et al.* (1997) *Int. J. Cancer.* 71: 842-850.

APC protein is expressed by the colorectal cancer cell line SW480 (available from the ATCC) as described by Munemitsu, S. *et al.* (1995) *PNAS* 92: 3046-3050.

CLAIMS:

1. A method of detecting the immune response of a mammal to circulating tumour marker proteins or tumour cells expressing said tumour marker proteins, which method comprises steps of:

(a) contacting a sample of bodily fluids from said mammal with a panel of two or more distinct tumour marker antigens;

(b) determining the presence or absence of complexes of said tumour marker antigens bound to autoantibodies present in said sample of bodily fluids, said autoantibodies being immunologically specific to said tumour marker proteins;

whereby the presence of said complexes is indicative of the immune response to circulating tumour marker proteins or tumour cells expressing said tumour marker proteins.

2. A method as claimed in claim 1 wherein at least one of said two or more tumour marker antigens is labelled with a protein or peptide tag.

3. A method as claimed in claim 1 or claim 2 wherein at least one of said tumour marker antigens is labelled with biotin.

4. A method as claimed in any of claims 1 to 3 wherein at least one of said two or more tumour marker antigens is selected from MUC1, p53, c-erbB2, Ras, c-

myc, BRCA1, BRCA2, PSA, APC or CA125 .

5. A method as claimed in claim 4 wherein the autoantibodies detected are indicative of cancer, preferably breast, bladder, colorectal, prostate or ovarian cancer.

6. A method as claimed in claim 4 or claim 5 wherein said panel comprises at least p53 and c-erbB2.

7. A method as claimed in claim 6 wherein the cancer is bladder cancer and the panel also includes MUC1 and/or c-myc.

8. A method as claimed in claim 6 wherein the cancer is colorectal cancer and the panel also includes Ras and/or APC.

9. A method as claimed in claim 6 wherein the cancer is prostate cancer and the panel also includes PSA and/or BRCA1.

10. A method as claimed in claim 6 wherein the cancer is breast cancer and the panel also includes MUC1 and/or c-myc and/or BRCA1 and/or BRCA2 and/or PSA

11. A method as claimed in claim 6 wherein the cancer is ovarian cancer and the panel also includes BRCA1 and/or CA125.

12. A method as claimed in claim 5 wherein the cancer is colorectal cancer and the panel is selected from the following:
p53 and Ras optionally with c-erbB2 and/or APC,

p53 and APC optionally with c-erbB2 and/or Ras, or Ras and APC optionally with p53 and/or c-erbB2.

13. A method as claimed in claim 5 wherein the cancer is prostate cancer and the panel is selected from the following:
p53 and PSA optionally with BRCA1 and/or c-erbB2, or c-erbB2 and PSA optionally with p53 and/or BRCA1.

14. A method as claimed in claim 5 wherein the cancer is ovarian cancer and the panel is selected from the following:
p53 and CA125 optionally with c-erbB2 and/or BRCA1, or c-erbB2 and CA125 optionally with p53 and/or BRCA1.

15. A method as claimed in claim 5 wherein the cancer is breast cancer and the panel is selected from the following:
p53 and MUC 1 with optional c-erbB2 and/or c-myc, and/or BRCA1 and/or BRCA2 and/or PSA,
p53 and c-myc with optional c-erbB2 and/or MUC1 and/or BRCA1 and/or BRCA2 and/or PSA,
p53 and BRCA1 with optional c-erB2 and/or MUC 1 and/or c-myc and/or BRCA2 and/or PSA,
p53 and BRCA2 with optional c-erbB2 and/or MUC 1 and/or c-myc and/or BRCA1 and/or PSA,
c-erbB2 and MUC 1 with optional p53 and/or c-myc, and/or BRCA1 and/or BRCA2 and/or PSA,
c-erbB2 and c-myc with optional p53 and/or MUC1 and/or BRCA1 and/or BRCA2 and/or PSA,
c-erbB2 and BRCA1 with optional p53 and/or MUC 1 and/or c-myc and/or BRCA2 and/or PSA, or
c-erbB2 and BRCA2 with optional p53 and/or MUC 1 and/or c-myc and/or BRCA1 and/or PSA.

16. An assay kit suitable for performing the assay method as claimed in any preceding claim, said assay kit comprising a panel of two or more distinct tumour marker antigens and means for contacting said panel with a sample of bodily fluids.

17. Use of the method of any of claims 1 to 15 in the detection of cancer, preferably breast, bladder, colorectal, prostate or ovarian cancer.

18. Use of the method of any of claims 1 to 15 in the detection of early neoplastic or early carcinogenic change in asymptomatic patients.

19. Use of the method of any of claims 1 to 15 in the detection of recurrent disease in a patient previously diagnosed as carrying tumour cells, which patient has undergone treatment to reduce the number of said tumour cells.

20. Use of the method of any of claims 1 to 15 in the identification of those individuals which are at increased risk of developing cancer in a population of asymptomatic individuals.

21. The use as claimed in claim 20 in which those individuals are at increased risk of developing breast cancer, bladder cancer, prostate cancer, colorectal cancer or ovarian cancer.

22. Use of the method of any of claims 1 to 15 in determination of the tumour marker profile of an individual suffering from cancer.

23. The use as claimed in claim 19 wherein said tumour marker profile is determined sequentially in said individual as an indication of the course of disease.

24. Use of the method of any of claims 1 to 15 in predicting the response of an individual with cancer to anti-cancer treatment.

25. The use as claimed in claim 24 wherein said anti-cancer treatment is hormone therapy, chemotherapy, radiotherapy, anti-growth factor therapy, immune therapy or vaccination.

26. A method of determining the immune response of a patient to two or more circulating tumour marker proteins or to tumour cells expressing said tumour marker proteins and identifying which one of said two or more tumour marker proteins elicits the strongest immune response in said patient, which method comprises steps of:

(a) contacting a sample of bodily fluids from said patient with a panel of two or more distinct tumour marker antigens;

(b) measuring the amount of complexes formed by binding of each of said tumour marker antigens to autoantibodies present in said sample of bodily fluids, said autoantibodies being immunologically specific to said tumour marker proteins;

(c) using the measurement obtained in part (b)

as an indicator of the relative strength of the immune response to each tumour marker protein and thereby identifying which one of said two or more tumour marker proteins elicits the strongest immune response in said patient.

27. A method as claimed in claim 23 wherein at least one of said tumour marker antigens is labelled with a protein or peptide tag.

28. A method as claimed in claim 25 or claim 26 wherein at least one of said tumour marker antigens is labelled with biotin.

29. A method as claimed in any of claims 26 to 28 wherein at least one of said tumour marker antigens is selected from MUC1, c-erbB2, c-myc, Ras, p53, BRCA1, BRCA2, PSA, APC or CA125.

30. Use of the method of any of claims 26 to 28 in the selection of a course of vaccine treatment.

31. Use as claimed in claim 30 wherein one or more tumour marker proteins identified as eliciting a strong immune response in said patient is/are used as the basis of a vaccine treatment.

32. A preparation comprising a human MUC1 protein, wherein said MUC1 manifests all the antigenic characteristics of a MUC1 protein obtainable from the bodily fluids of a patient with advanced breast cancer.

33. A preparation as claimed in claim 32, wherein the MUC1 protein exhibits altered affinity for the antibodies B55, C595, BC4W154, DF3, B27.29, 115D8, 27.1, SM3, Ma552, HMPV and BC2 compared to MUC1 protein isolated from normal human urine.

34. A preparation as claimed in claim 32 or claim 33 wherein the MUC1 protein has an affinity profile with the antibodies B55, C595, BC4W154, DF3, B27.29, 115D8, 27.1, SM3, Ma552, HMPV and BC2 substantially identical to that shown in Figure 2.

35. A preparation as claimed in any one of claims 32 to 34 wherein the MUC1 protein is isolated from the serum of one or more human patients with breast cancer, preferably advanced breast cancer or primary breast cancer.

36. A tumour marker protein substantially equivalent to human MUC1 protein, which tumour marker protein is isolatable from the serum of one or more patients with advanced breast cancer and which tumour marker protein is characterised by exhibiting affinity for the antibody 115D8 3 to 6 times greater than the affinity of MUC1 protein isolated from normal human urine for said antibody.

37. A method of detecting or quantitatively measuring autoantibodies immunologically specific to MUC1 protein, which method comprises the steps of:

(a) contacting a sample to be tested for said autoantibodies with the tumour marker protein of any one of claims 32 to 36;

(b) determining the presence or absence of complexes of said tumour marker protein bound to said autoantibodies.

38. Use of the method of claim 37 in the identification of those individuals which are at risk of developing breast cancer or bladder cancer in a population of asymptomatic individuals.

39. Use of the method of claim 37 in the detection of cancer.

40. Use of the method of claim 37 in the detection of recurrent breast cancer in a patient previously diagnosed as carrying breast cancer cells, which patient has undergone treatment to reduce the number of said breast cancer cells.

41. Use of the method of claim 37 in the detection of early neoplastic or early carcinogenic change in asymptomatic patients.

42. A method of quantifying the immune response of a mammal to circulating tumour marker proteins or tumour cells expressing said tumour marker proteins wherein at least one of said tumour marker protein is c-erbB2, Ras, c-myc, p53, BRCA1, BRCA2, APC, PSA or CA125

which method comprises steps of:

(a) contacting a sample of bodily fluids with at least one tumour marker antigen selected

from c-erbB2, ras, biotinylated c-myc, BRCA1, BRCA2, APC, PSA, CA125 or biotinylated p53;

(b) measuring the quantity of complexes formed by binding of said at least one tumour marker antigen to autoantibodies present in said sample of bodily fluids, said autoantibodies being immunologically specific to said tumour marker protein; and

(c) using the measurement obtained in (b) as a indicator of the amount of said autoantibodies present in said sample.

43. A method as claimed in claim 42 wherein said tumour marker antigen is labelled with a protein or peptide tag.

44. A method as claimed in claim 42 or claim 43 wherein said tumour marker is labelled with biotin.

45. An assay kit suitable for performing the method of any one of claims 42 to 44, said assay kit comprising said tumour marker antigen, means for contacting said antigen with said sample of bodily fluids and means for facilitating quantitative assessment of said complexes formed by binding of said at least one tumour marker antigen to autoantibodies present in said sample of bodily fluids.

46. Use of the method of any one of claims 42 to 44 in the detection of cancer.

47. Use as claimed in claim 46 wherein the cancer is breast, colorectal, bladder, prostate or ovarian cancer.

48. Use of the method of any one of claims 42 to 44 in the detection of early neoplastic or early carcinogenic change in asymptomatic patients.

49. Use of the method of any one of claims 42 to 44 in the detection of recurrent disease in a patient previously diagnosed as carrying tumour cells, which patient has undergone treatment to reduce number of said tumour cells.

50. Use of the method of any one of claims 42 to 44 in monitoring the progress of neoplastic disease.

51. Use of the method of any one of claims 42 to 44 in the identification of those individuals which are at increased risk of developing cancer in a population of asymptomatic individuals.

52. The use as claimed in claim 51 wherein those individuals are at increased risk of developing breast cancer, colorectal cancer, bladder cancer, prostate cancer or ovarian cancer.

53. Use of the method of any one of claims 42 to 44 in predicting and/or measuring the response of an individual with cancer to anti-cancer treatment.

54. The use as claimed in claim 53 wherein the anti-cancer treatment is hormone therapy, chemotherapy, radiotherapy, anti-growth factor

therapy, immune therapy or vaccination.

55. A method of detecting the immune response of a mammal to a circulating tumour marker protein or tumour cells expressing said tumour marker protein wherein said tumour marker protein is any one of the tumour marker proteins listed in claim 4, said method comprising the steps of:

(a) contacting a sample of bodily fluids from said mammal with MUC1, c-erbB2, Ras, biotinylated c-myc, biotinylated p53, BRCA1, BRCA2, APC, PSA or CA125 or an antigenic fragment thereof;

(b) determining the presence or absence of complexes of said tumour marker protein or antigenic fragment thereof bound to autoantibodies present in said sample of bodily fluids, said autoantibodies being immunologically specific to said tumour marker protein or antigenic fragment thereof;

whereby the presence of said complexes is indicative of the immune response to said circulating tumour marker protein or tumour cells, expressing said tumour marker protein.

56. A method as claimed in claim 55 wherein said tumour marker antigen is labelled with a protein or peptide tag.

57. A method as claimed in claim 55 or claim 66

wherein said tumour marker antigen is labelled with biotin.

58. Use of the method of any one of claims 55 to 57 in the detection of cancer.

59. Use as claimed in claim 58 wherein the cancer is breast, colorectal, bladder, prostate or ovarian cancer.

60. Use of the method of any one of claims 55 to 57 in the detection of early neoplastic or early carcinogenic change in asymptomatic patients.

61. Use of the method of any one of claims 55 to 57 in the detection of recurrent disease in a patient previously diagnosed as carrying tumour cells, which patient has undergone treatment to reduce the number of said tumour cells.

62. Use of the method of any one of claims 55 to 57 in monitoring the progress of cancer or other neoplastic disease.

63. Use of the method of any one of claims 55 to 57 in the identification of those individuals which are at increased risk of developing cancer in a population of asymptomatic individuals.

64. The use as claimed in claim 63 wherein those individuals are at increased risk of developing breast cancer, colorectal cancer, bladder cancer, prostate cancer or ovarian cancer.

65. Use of the method of any one of claims 55 to 57 in predicting and/or the response of an individual with cancer to anti-cancer treatment.

66. The use as claimed in claim 65 wherein the anti-cancer treatment is hormone therapy, chemotherapy, radiotherapy, anti-growth factor therapy, immune therapy or vaccination.

67. A method of determining whether a vaccination procedure comprising challenging a patient with an immunogenic preparation comprising MUC1 protein or an antigenic fragment thereof has been successful in eliciting a humoral immune response, which method comprises the steps of:

(a) contacting a sample of serum from said patient with the modified MUC1 protein of any one of claims 32 to 36;

(b) determining the presence or absence of complexes of said modified MUC1 protein bound to antibodies present in said sample of serum, said antibodies being immunologically specific to MUC1 or an antigenic fragment thereof

whereby the presence of said complexes indicates that said vaccination procedure has been successful in eliciting a humoral immune response.

68. A method of detecting recurrent disease in a patient previously diagnosed as carrying tumour cells,

which patient has undergone treatment to reduce the number of said tumour cells, which method comprises steps of:

(a) contacting a sample of bodily fluids from said patient with MUC1 protein or an antigenic fragment thereof;

(b) determining the presence or absence of complexes of said MUC1 protein or antigenic fragment thereof bound to autoantibodies present in said sample of sample of bodily fluids, said autoantibodies being immunologically specific to MUC1.

whereby the presence of said complexes indicates the presence of recurrent disease in said patient.

69. A method as claimed in claim 68 wherein said patient has been diagnosed with primary breast cancer prior to treatment to reduce the number of said tumour cells.

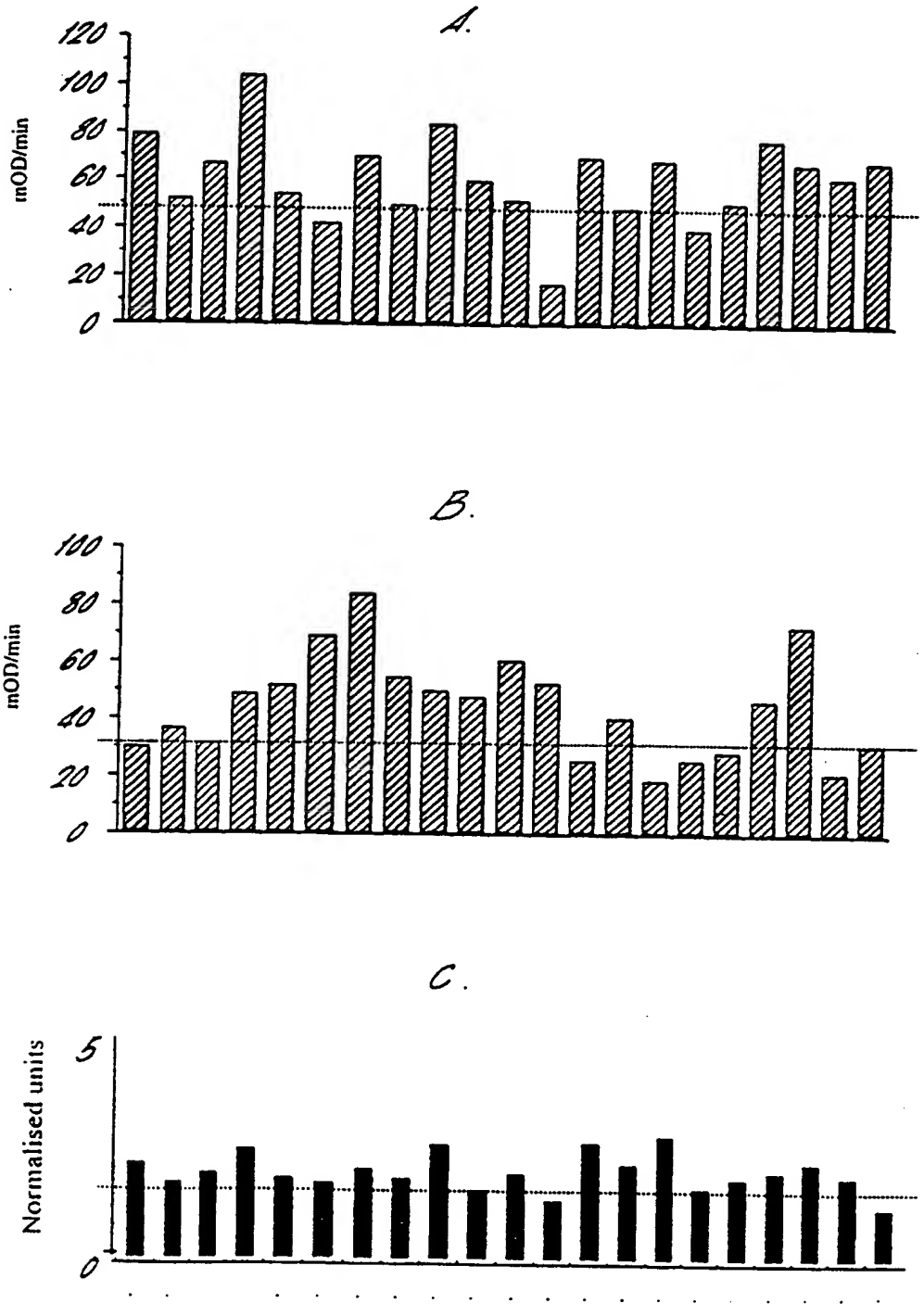
70. Use of the method of claim 37 for selection of appropriate vaccine treatment.

71. Use of the method of claim 37 for predicting and/or measuring the response of an individual with cancer to anti-cancer treatment.

72. The use as claimed in claim 71 wherein the anti-cancer treatment is hormone therapy, chemotherapy, radiotherapy, anti-growth factor

therapy, immune therapy or vaccination

FIG. 1.


A.
120
100
80
60
40
20
0
mOD/min
B.
100
80
60
40
20
0
mOD/min
C.
5
0
Normalised units

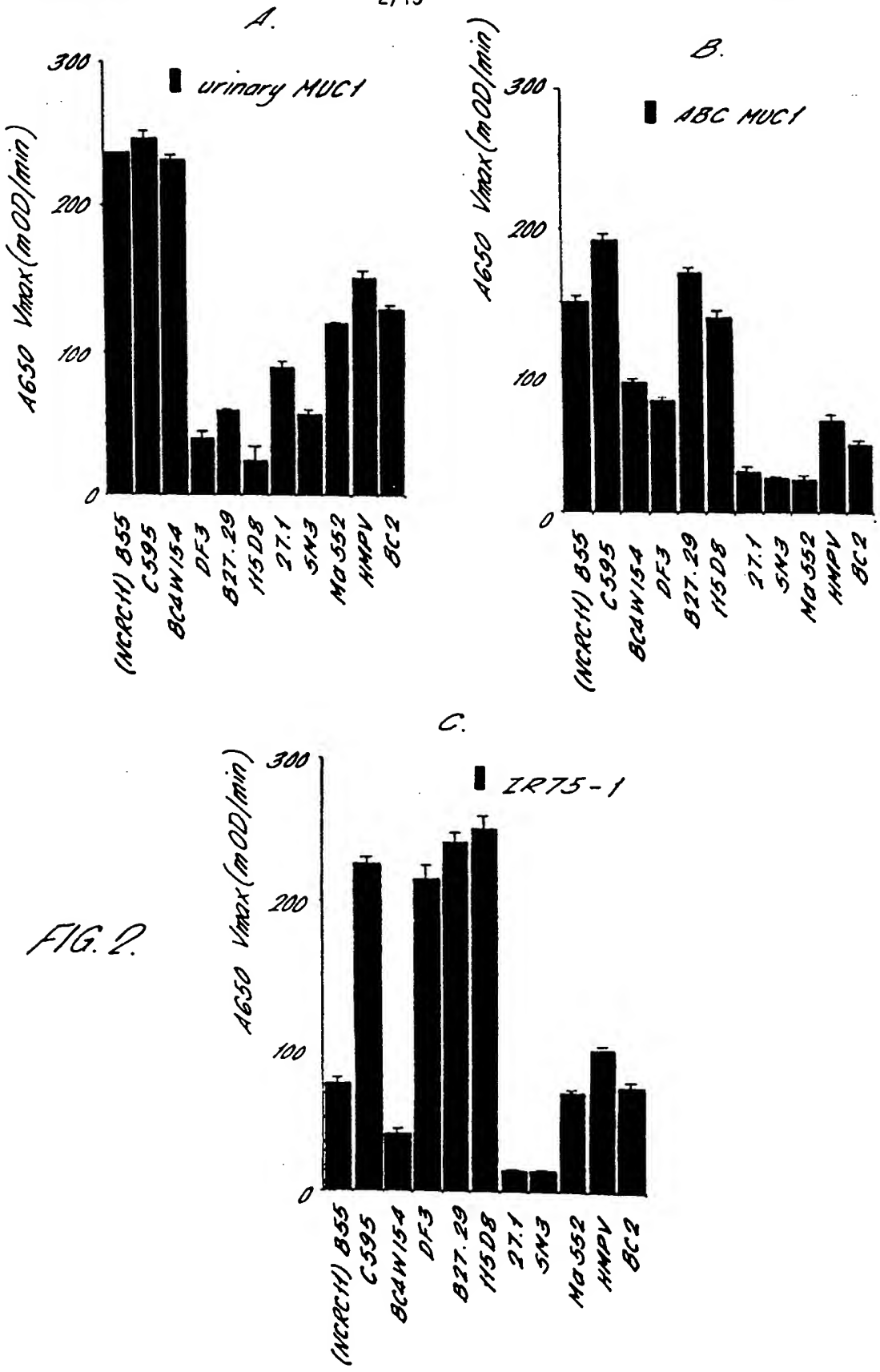

A.
urinary MUC1
A650 Vmax (mOD/min)
300
200
100
0
(NCRCH) B55
C595
BC4W154
DF3
B27.29
115D8
27.1
SN3
Mo552
HMPV
BC2
B.
ABC MUC1
A650 Vmax (mOD/min)
300
200
100
0
(NCRCH) B55
C595
BC4W154
DF3
B27.29
115D8
27.1
SN3
Mo552
HMPV
BC2
C.
IR75-1
A650 Vmax (mOD/min)
300
200
100
0
(NCRCH) B55
C595
BC4W154
DF3
B27.29
115D8
27.1
SN3
Mo552
HMPV
BC2

FIG. 2.

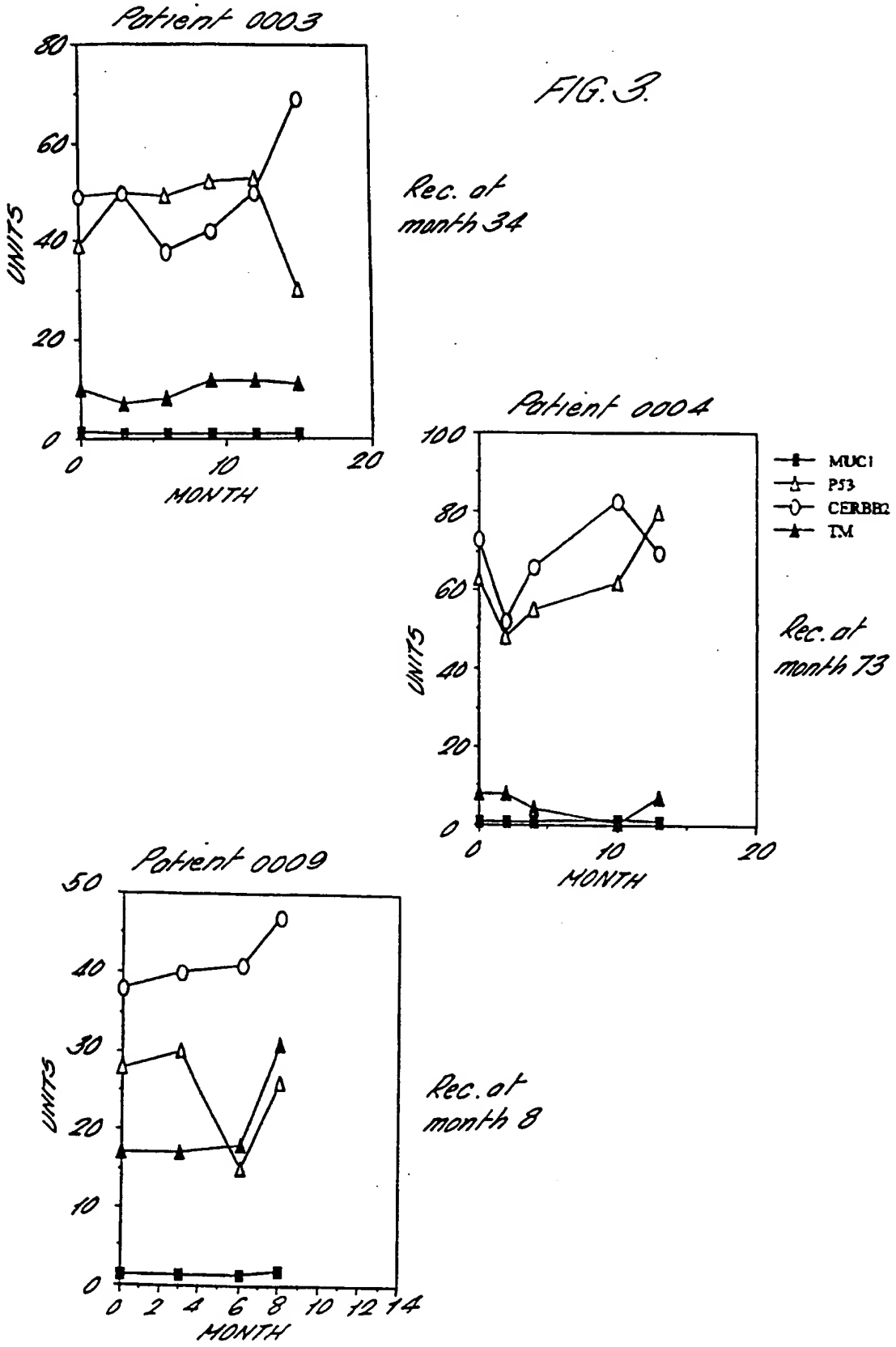

Patient 0003
UNITS
MONTH
Rec. at month 34
Patient 0004
UNITS
MONTH
MUC1
P53
CERBB2
TM
Rec. at month 73
Patient 0009
UNITS
MONTH
Rec. at month 8

FIG. 3.

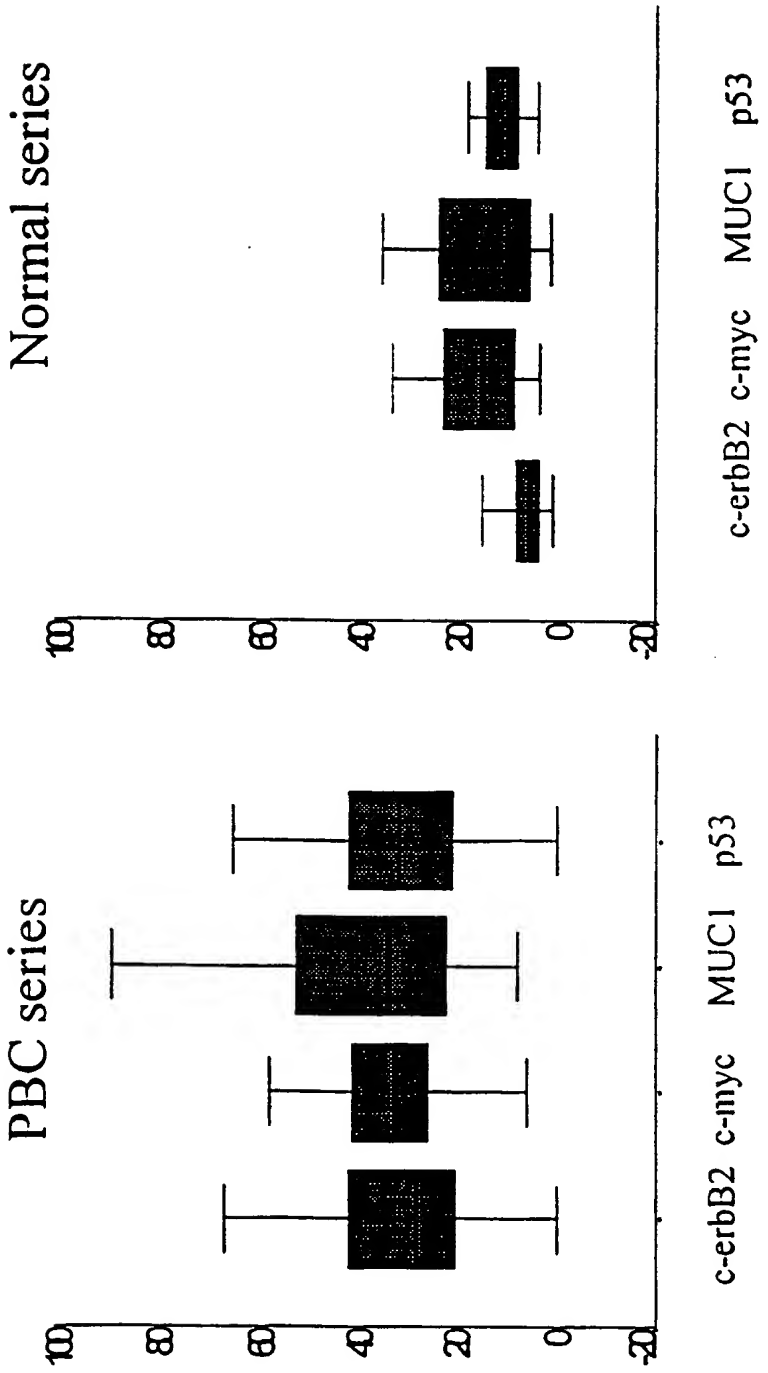

Normal series
PBC series
100
80
60
40
20
0
-20
c-erbB2
c-myc
MUC1
p53

FIG. 4.

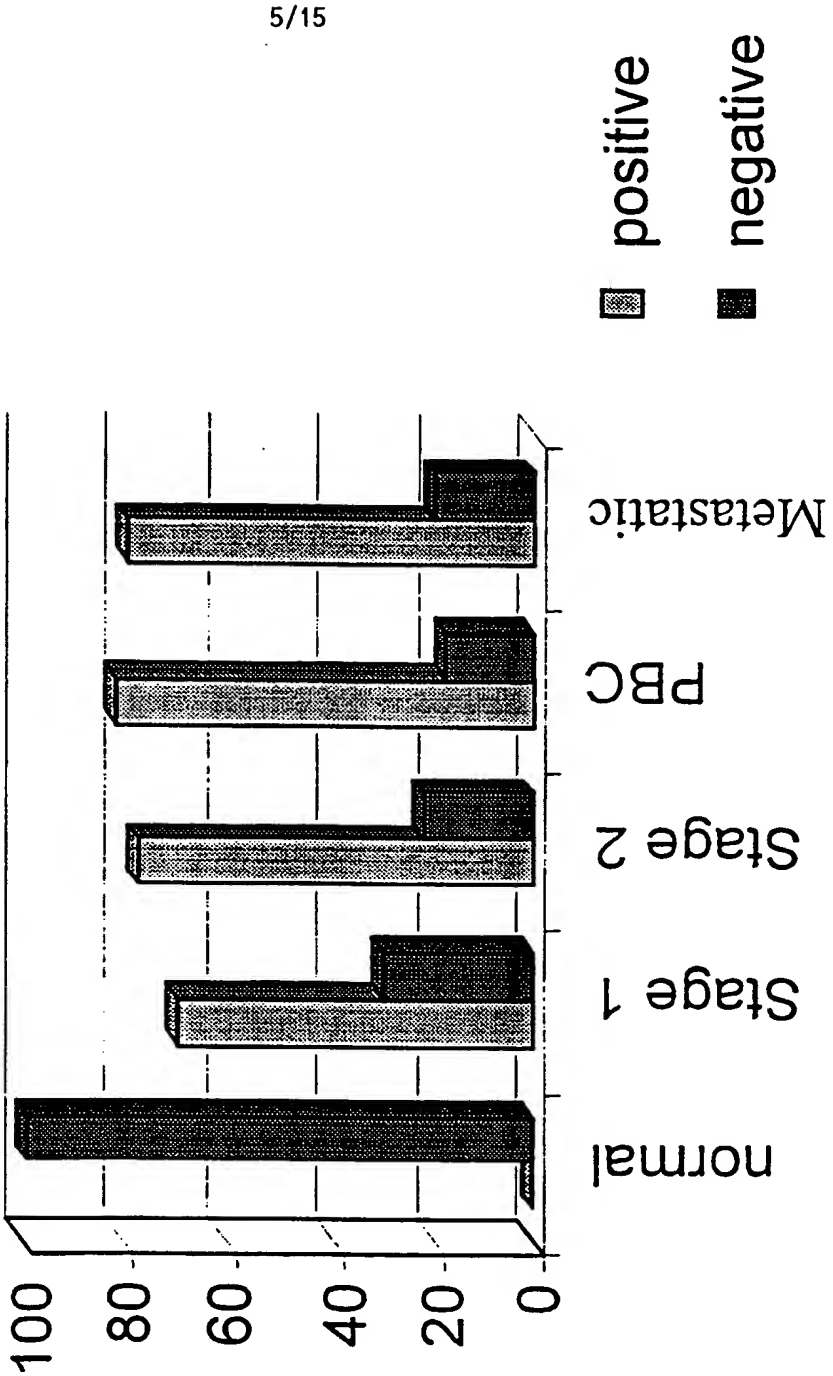

positive
negative
normal
Stage 1
Stage 2
PBC
Metastatic
0
20
40
60
80
100

FIG.5.

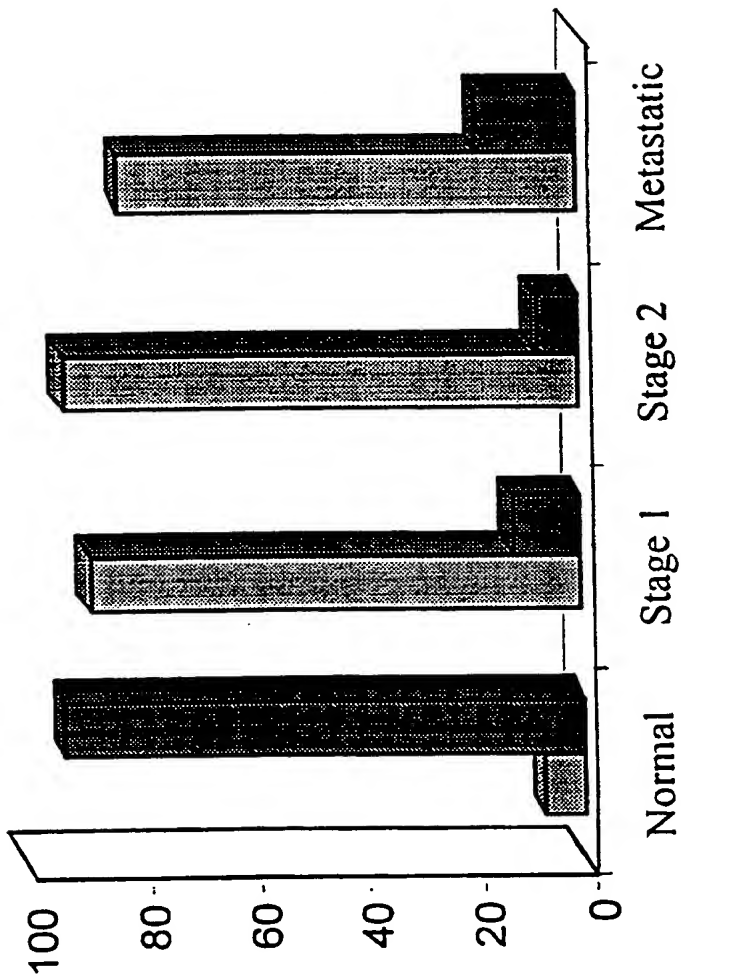

positive
negative
100
80
60
40
20
0
Normal
Stage 1
Stage 2
Metastatic

FIG. 6.

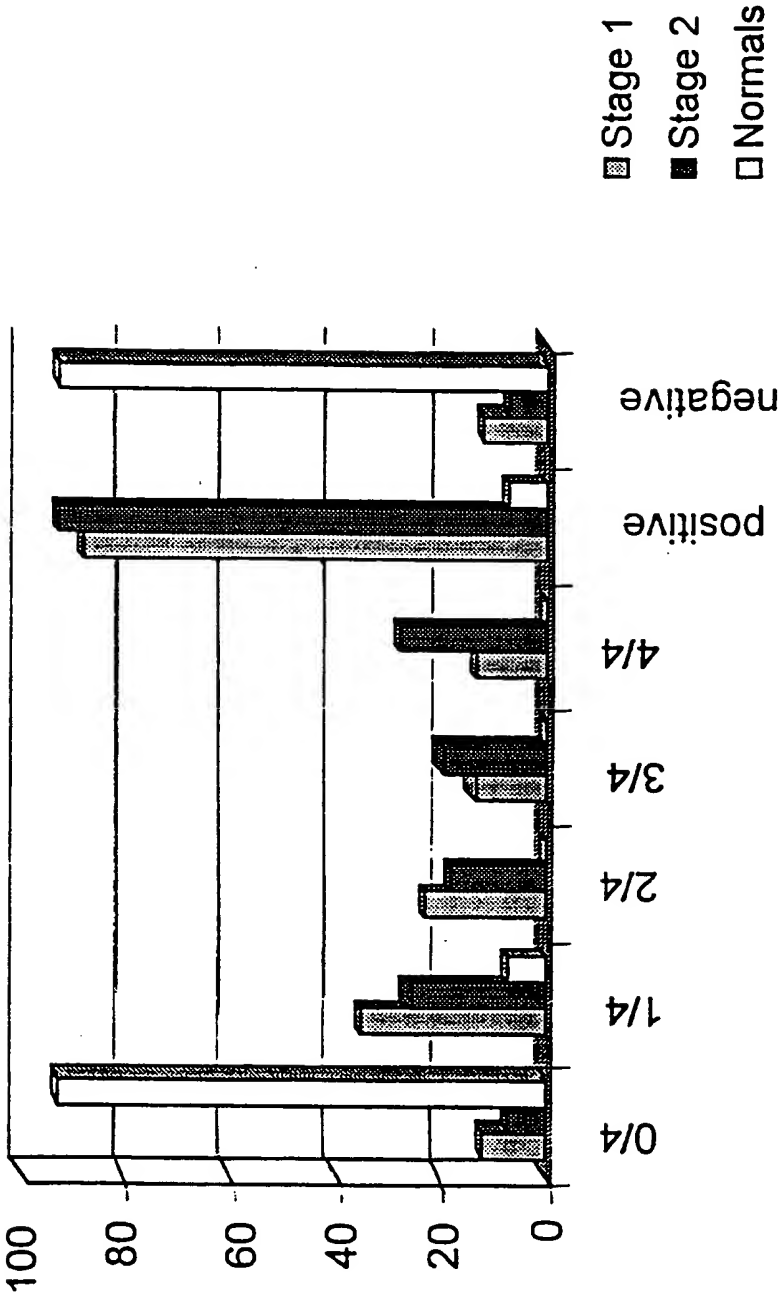

Stage 1
Stage 2
Normals
negative
positive
4/4
3/4
2/4
1/4
0/4
100
80
60
40
20
0

FIG. 7.

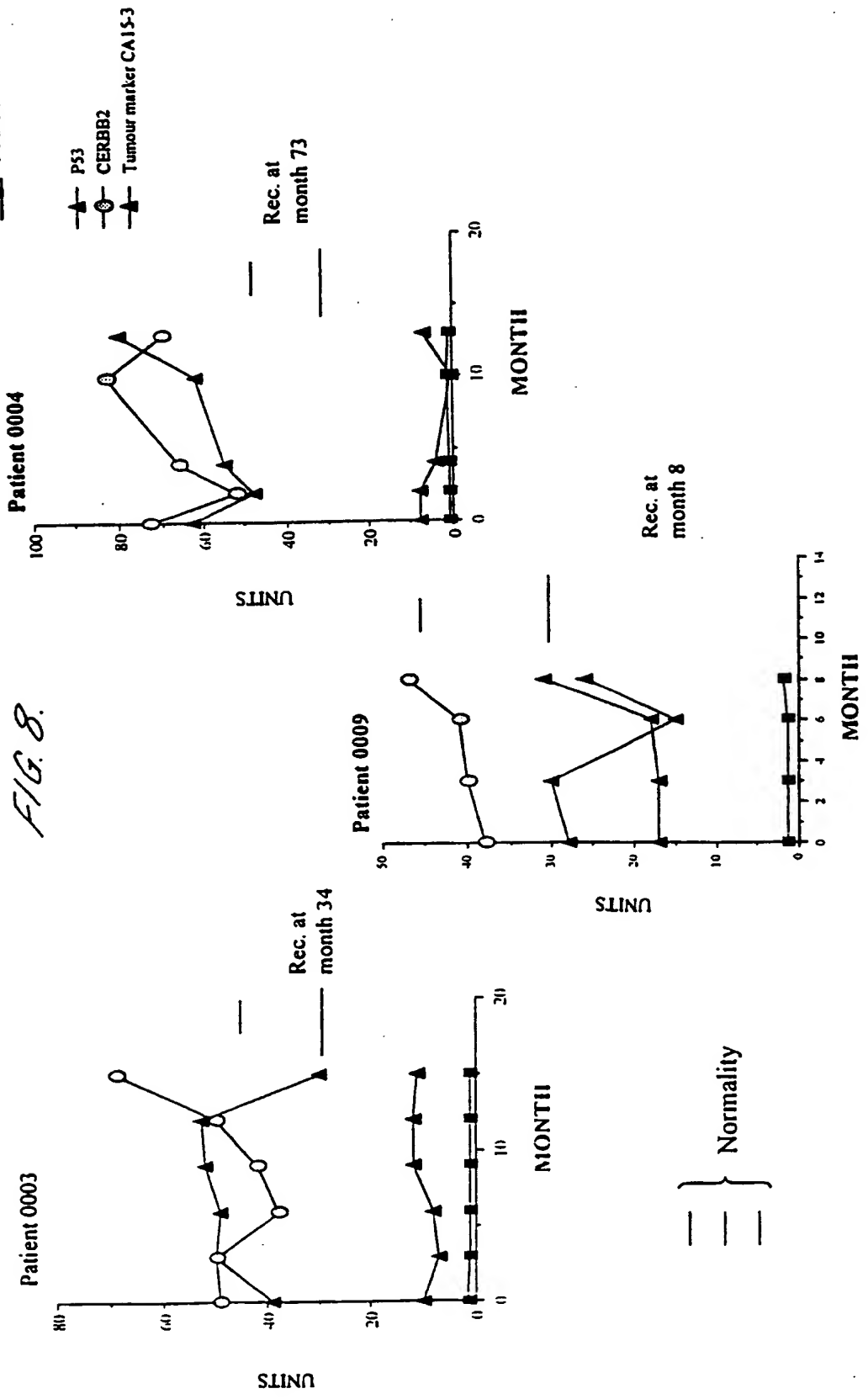

MUC1
P53
CERBB2
Tumour marker CA15-3
Patient 0004
Rec. at month 73
UNITS
MONTH
Patient 0009
Rec. at month 8
Patient 0003
Rec. at month 34
Normality

FIG. 8.

FIG. 9

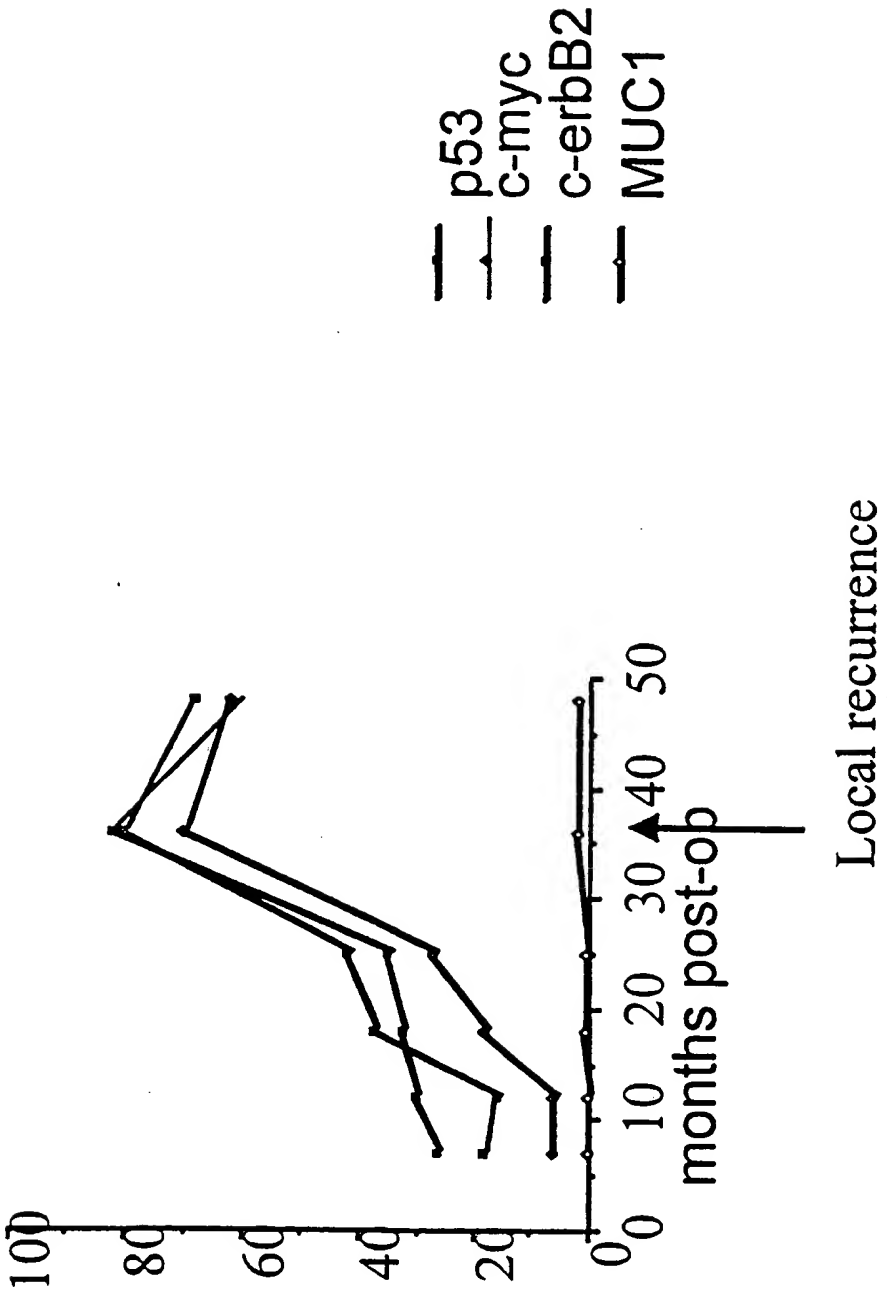
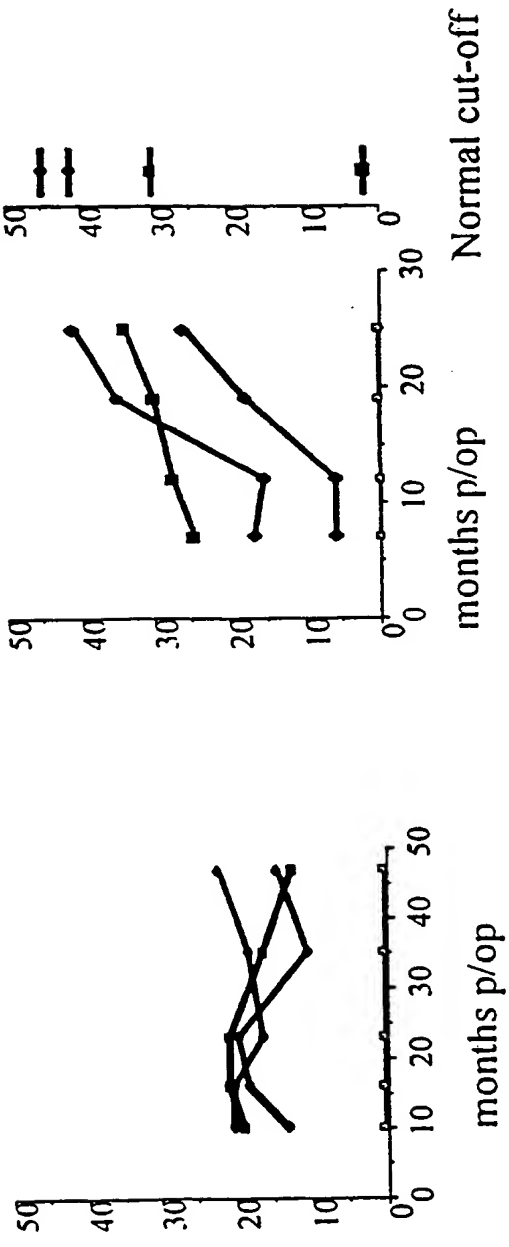

p53
c-myc
c-erbB2
MUC1
100
80
60
40
20
0
0
10
20
30
40
50
months post-op
Local recurrence

FIG. 10.


50
40
30
20
10
0
0 10 20 30 40 50
months p/op
No Rec.
0 10 20 30
months p/op
Rec. at 36/12
Normal cut-off
MUC1
p53
c-erbB2
c-myc

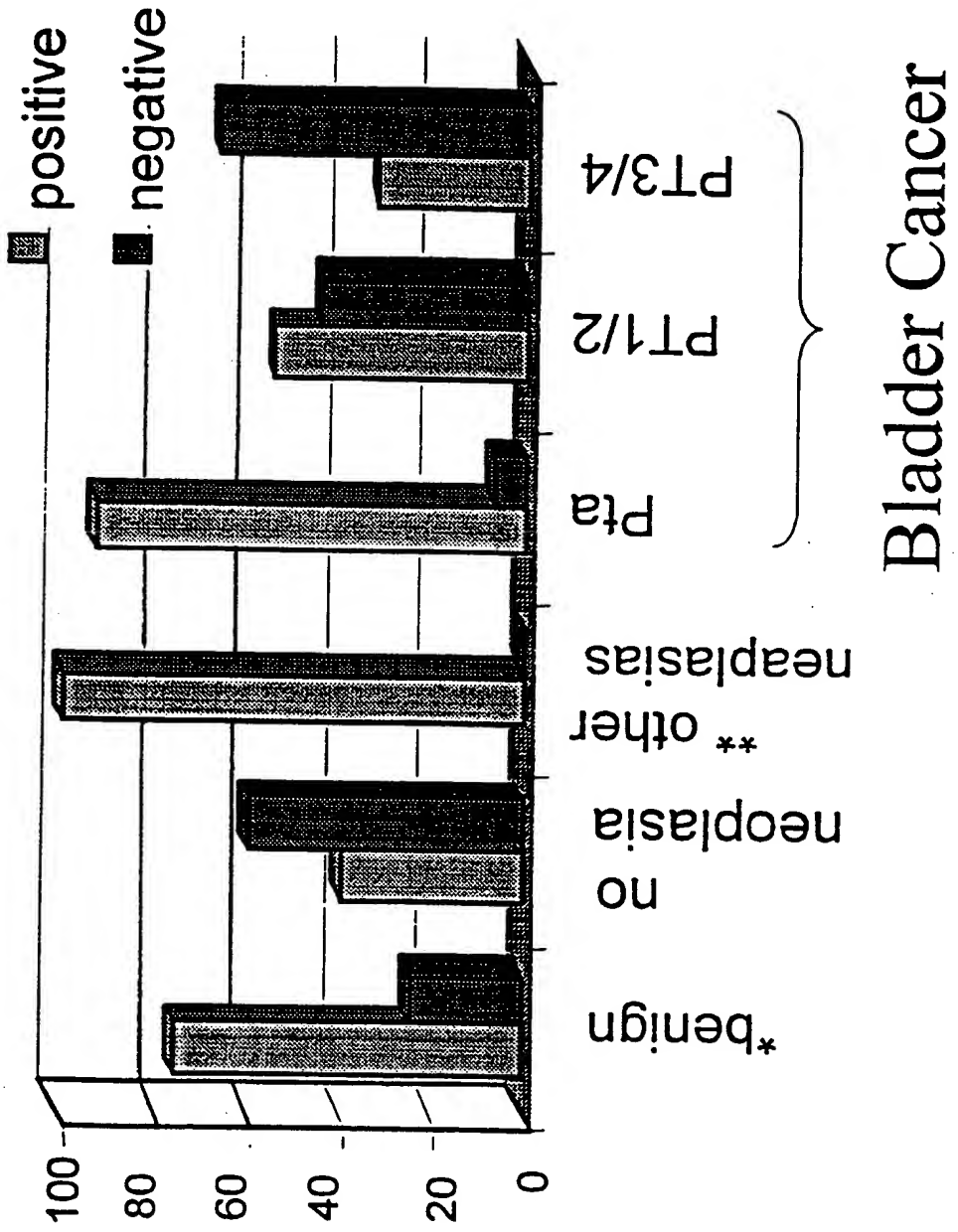

positive
negative
100
80
60
40
20
0
*benign
no neoplasia
** other neoplasias
Pta
PT1/2
PT3/4
Bladder Cancer

FIG. 11.

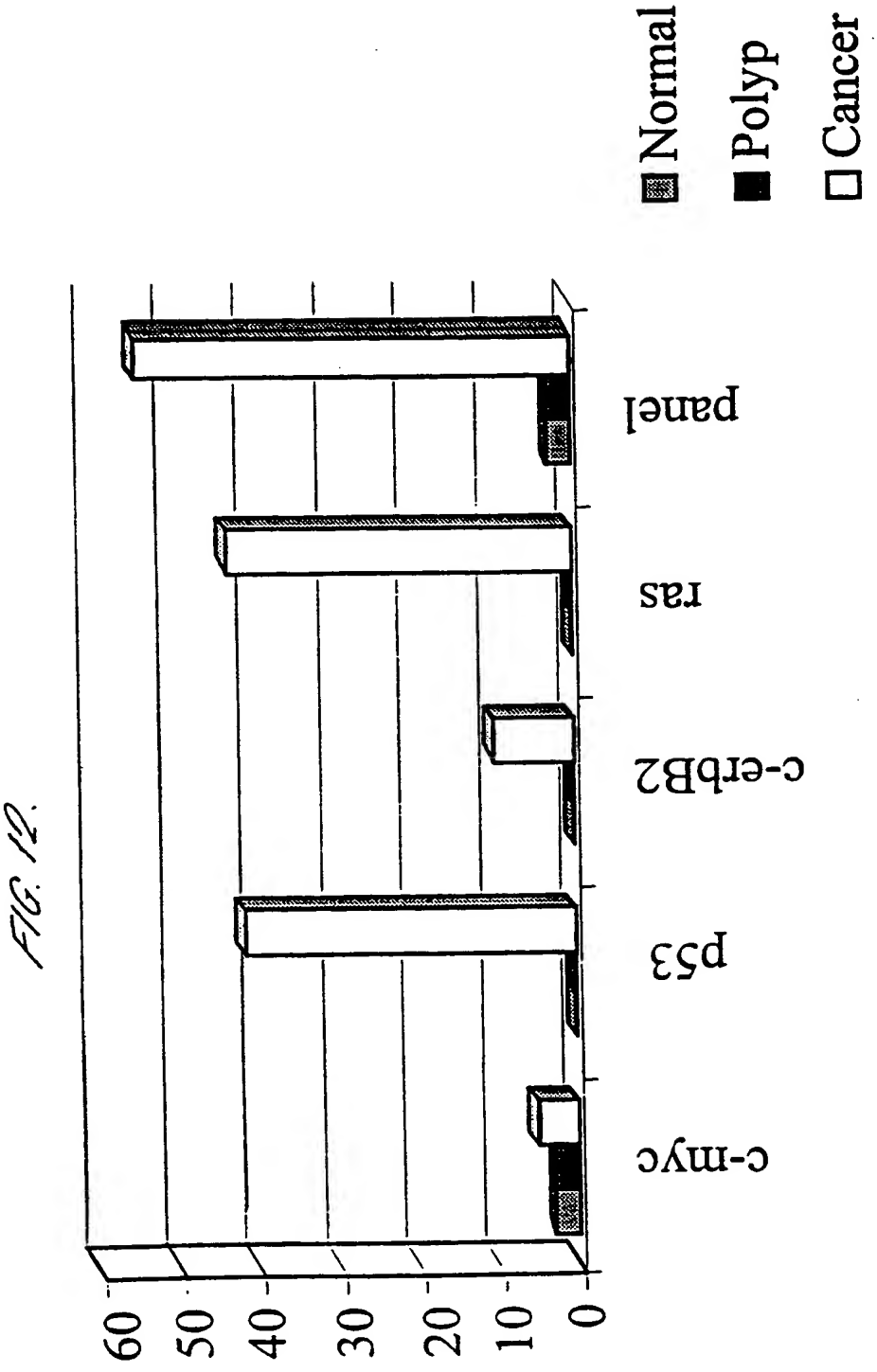

Normal
Polyp
Cancer
panel
ras
c-erbB2
p53
c-myc
60
50
40
30
20
10
0

FIG. 12.

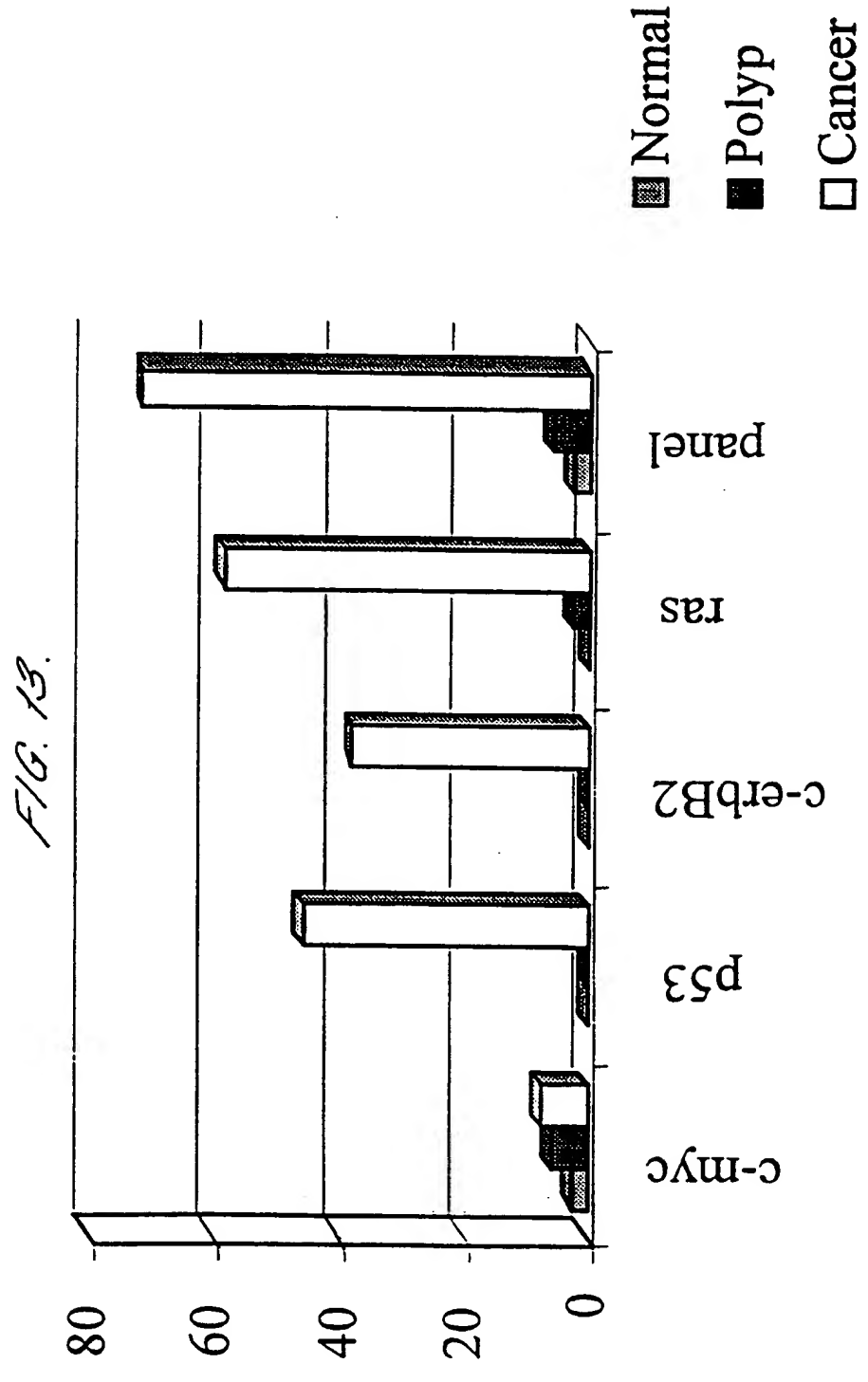


FIG. 13.

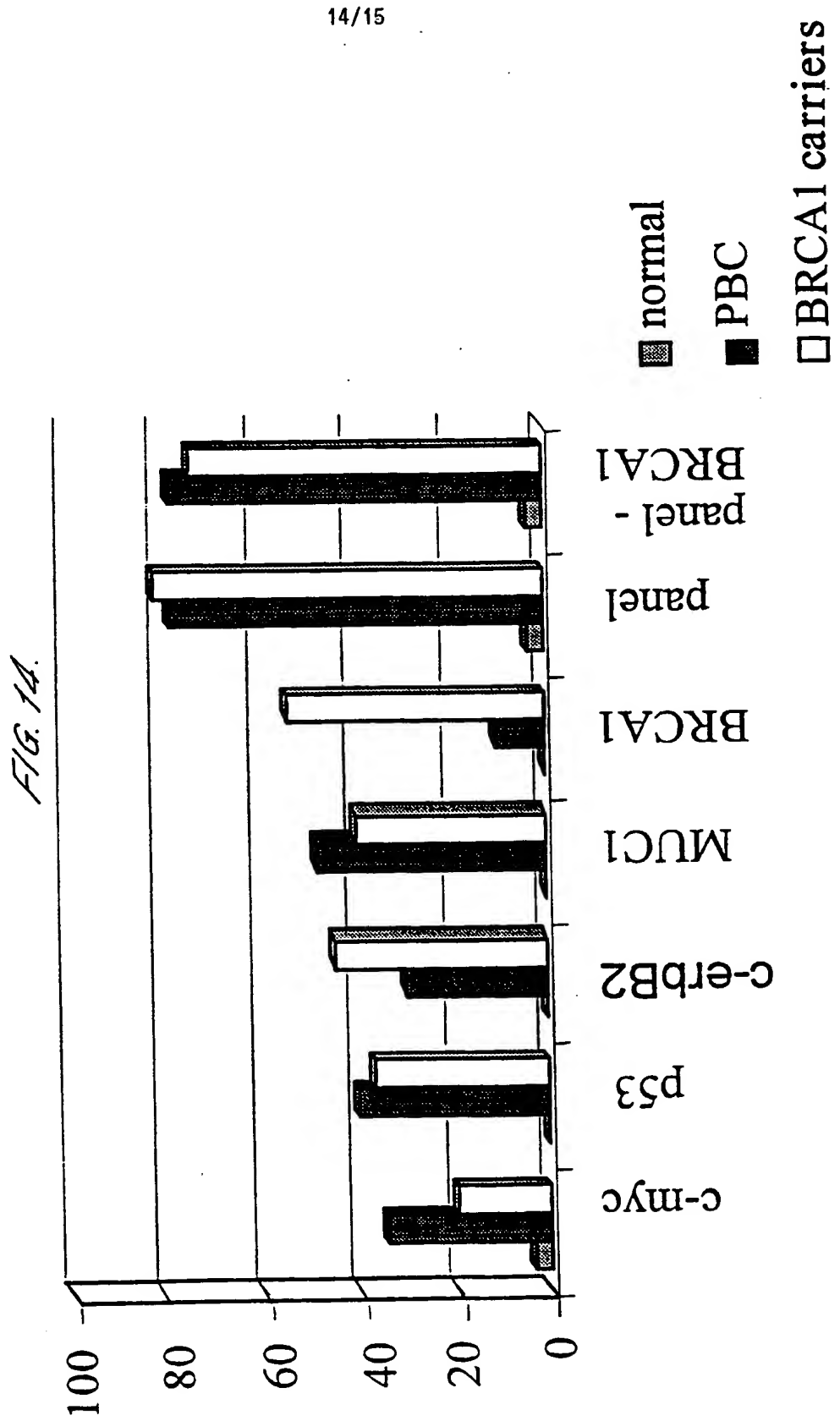

normal
PBC
BRCA1 carriers
c-myc
p53
c-erbB2
MUC1
BRCA1
panel
panel - BRCA1
0
20
40
60
80
100

FIG. 14.

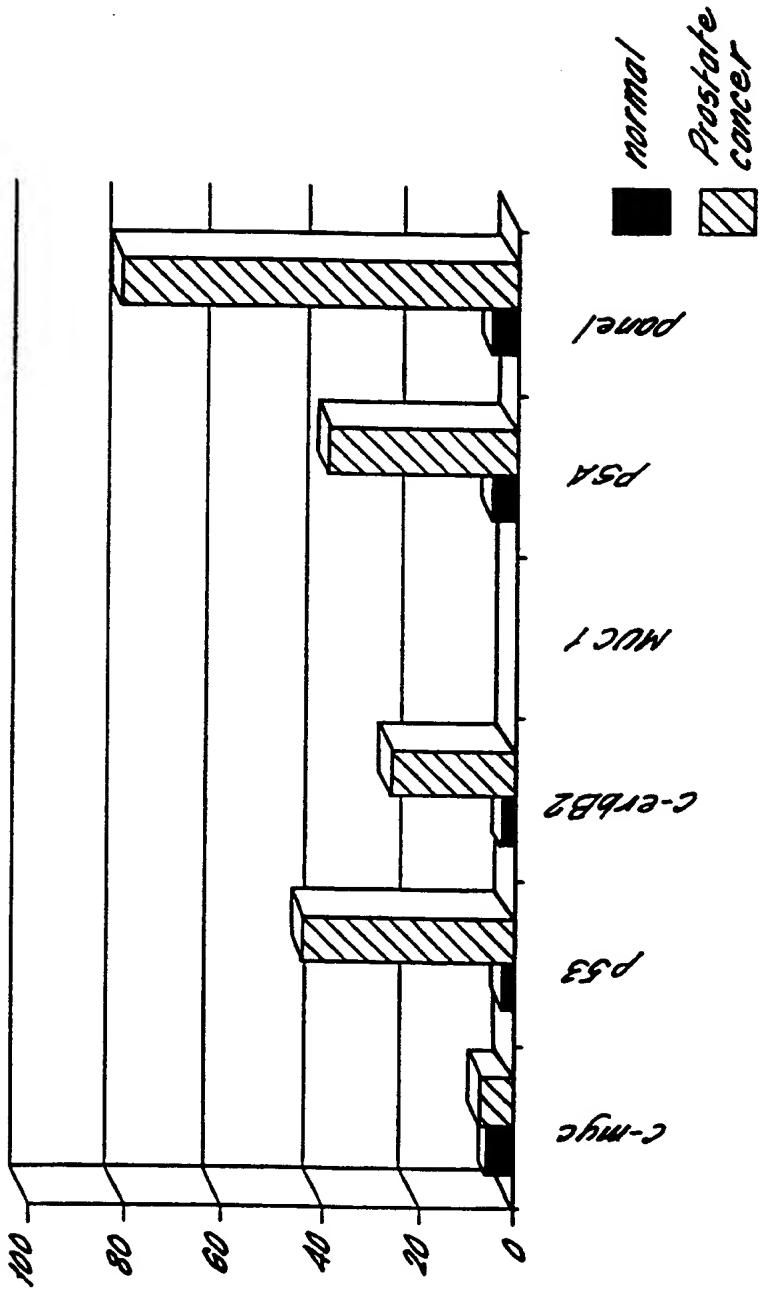

normal
Prostate cancer
PSMA
PSA
MUC1
c-erbB2
p53
c-myc
100
80
60
40
20
0

FIG. 15.

JOINT INTEREST CONFIDENTIALITY AGREEMENT

This Joint Interest Confidentiality Agreement ("Agreement") is entered into by and among: (1) Biotec Pharmacon ASA, Strandgata 3, N-9008 Tromsø, Norway ("Biotec"); (2) Immunocorp, 1528 W. Deere Ave., Irvine, CA ("Immunocorp"); and (3) Associated British Foods, PLC XXXX ("ABF"), (Biotec, Immunocorp, and ABF are collectively referred to as the "Parties"). The Parties have entered into this Agreement on their own behalf and on behalf of their respective counsel, affiliates or agents, who are assigned to assist, review, or otherwise participate in the circumstances set forth herein. This Agreement is expressly made *nunc pro tunc* to have the same legal force and effect as if executed on _____.

RECITALS

ABF is a manufacturer of MacroGard Feed Ingredient ("MG-FI"), a yeast-derived beta glucan powder that is manufactured from ABF's proprietary strain of *Saccharomyces cerevisiae* ("ABF's Proprietary Yeast Strain").

Biotec is a supplier of yeast-derived beta glucan products, including MG-FI manufactured by ABF. Biotec also manufactures various yeast-derived beta glucan products from MG-FI as manufactured by ABF. Immunocorp is a United States subsidiary of Biotec.

Biotec and Immunocorp have been sued for patent infringement in the District Court for the District of Minnesota, Civil No. 05-536 JNE/SRN, by Biopolymer Engineering d/b/a/ Biothera. Biothera is a Minnesota corporation located at 3388 Mike Collins Drive, Eagan, Minnesota 55121. The lawsuit relates, at least in part, to MG-FI and ABF's Proprietary Yeast Strain.

In connection with the Parties' joint efforts to analyze and provide legal advice regarding ABF's Proprietary Yeast Strain and MG-FI, the Parties have concluded that confidential facts

and information known by each of them may assist each Party in the assessment of actual or potential litigation with Biothera. Thus, the Parties acknowledge and agree that they share certain common interests and that these common interests will be best served if their respective counsel can exchange information under the continued protection of the attorney-client, attorney work-product, and other privileges.

The Parties have been acting under an oral joint interest confidentiality agreement and wish to memorialize in this written agreement their preexisting understanding and agreement. By executing this Agreement, the Parties confirm that they wish to produce to each other information and documentation, both tangible and intangible, as is necessary to evaluate ABF's Proprietary Yeast Strain, MG-FI, and any actual or potential litigation with Biothera.

In order for the Parties to cooperate effectively, the Parties may share and expect to continue to share, on a privileged and confidential basis, certain legal research, mental impressions, opinions and advice of legal counsel regarding ABF's Proprietary Yeast Strain and MG-FI. The Parties understand and agree that all such privileged and confidential information, research, advice, legal opinions or reference to the same (the "Confidential Information") will be shared solely for the purposes of evaluating ABF's Proprietary Yeast Strain or MG-FI and assessing actual or potential claims of patent infringement and any actual or potential litigation with Biothera. The Parties wish to avoid the waiver of any privilege with respect to any otherwise privileged Confidential Information to, by or between counsel, including communications between counsel, between co-counsel and one or more of the Parties, or between the Parties (to the extent that one of those Parties is transmitting counsel's legal advice to any other Party).

Thus, in consideration of the mutual understandings and covenants in this Agreement and for other good and valuable consideration, the Parties agree:

1. Except as otherwise provided in this Agreement, the Parties will not disclose any Confidential Information received from any of the Parties, or any portion thereof, orally or in writing, to anyone other than officers, directors, auditors, employees, or attorneys of the Parties who need to know the same for purposes of evaluating ABF's Proprietary Yeast Strain or MG-FI. Disclosure to such persons will be made only if there is a bona fide need for the disclosure of Confidential Information. Furthermore, any disclosures to such persons will only be made with the express understanding and agreement that such persons will not further disseminate the Confidential Information, except as allowed by this Agreement.

2. Any person or entity receiving any Confidential Information under this Agreement will do so with the understanding that he or she will use the Confidential Information only for the express purpose of assessing and evaluating ABF's Proprietary Yeast Strain or MG-FI and assessing actual or potential claims of patent infringement and actual or potential litigation with Biothera. Each Party will refrain from any other use or disclosure of any Confidential Information without the express, written consent of the Parties. Nothing in this Agreement, however, will prevent any Party from disclosing to others the existence and substance of this Agreement.

3. At the request of Biotec and Immunocorp's counsel, ABF will provide a culture of ABF's Proprietary Yeast Strain to Biotec and Immunocorp's independent, scientific consultants for their testing and evaluation ("Evaluation"). ABF will also cooperate with Biotec and Immunocorp's independent, scientific consultants regarding the protocols or procedures

necessary to maintain ABF's Proprietary Yeast Strain in culture so that Biotec and Immunocorp's independent scientific consultants can complete their Evaluation.

Before receiving a culture of ABF's Proprietary Yeast Strain, however, each independent, scientific consultant must execute the Proprietary Yeast Strain Confidentiality Agreement, which is attached as **Exhibit A**, and provide an executed original of the Proprietary Yeast Strain Confidentiality Agreement to counsel for ABF. Any protocols or procedures concerning the maintenance of ABF's Proprietary Yeast Strain and any data or reports obtained from the Evaluation of ABF's Proprietary Yeast Strain will be treated as Confidential Information as described herein.

4. To date, counsel for Biothera has made no demands regarding ABF's Proprietary Yeast Strain. If, however, Biothera's counsel demands in writing that Biotec produce a culture of ABF's Proprietary Yeast Strain, ABF will produce such a culture to counsel for Biotec (for production to Biothera's counsel) or directly to counsel for Biothera. The Parties agree that any culture of ABF's Proprietary Yeast Strain produced to Biothera's counsel will be produced under the terms of the Biothera-Biotec-Immunocorp Stipulated Protective Order, a copy of which is attached as **Exhibit B**. Specifically, any culture of ABF's Proprietary Yeast Strain that is produced to counsel for Biothera must be clearly designated "CONFIDENTIAL—COUNSEL AND EXPERTS ONLY." Additionally, each of Biothera's independent, scientific consultants that receive such a culture must—in addition to executing Exhibit 1 of the Stipulated Protective Order—certify at the completion of the litigation that they have destroyed the culture by executing the affidavit attached as **Exhibit C**.

5. Nothing contained in this Agreement is to be construed as granting or conferring rights by license or otherwise in any Confidential Information disclosed to any of the Parties.

Any confidential, proprietary, or other trade secret Confidential Information shall remain the property of the party producing it.

6. Neither this Agreement nor any rights hereunder—in whole or in part—are assignable or otherwise transferable by the Parties. The confidentiality obligations contained in this Agreement will also survive and continue after termination of this Agreement.

7. The Parties acknowledge that they each have an important and legitimate interest in keeping confidential the Confidential Information that is the subject of this Agreement and that unauthorized use or disclosure of such Confidential Information may cause them irreparable and continuing harm. Consequently, in addition to such remedies as would be available at law, in the event of any breach of this Agreement by any Party or others receiving the Confidential Information, the Parties shall be entitled to enforce this Agreement through requests for equitable relief in addition to remedies at law.

8. If any Confidential Information held or known by any Party is sought by subpoena or other legal process, that Party shall promptly give written notice forwarding a copy of the legal process to each of the other Parties at the following addresses:

For Biotec and Immunocorp:

Alexander Bjorna
Strandgata 3, N-9008
Tromsø, Norway

Copy to:
Jason M. Pass
KILPATRICK STOCKTON LLP
1100 Peachtree Street
Suite 2800
Atlanta, Georgia 30309-4530

For ABF:

XXXXX
XXXXX

The Parties will oppose any disclosure of Confidential Information in connection with any such subpoena or other process by all reasonable legal means, unless disclosure is authorized by the Parties in writing. No Confidential Information will be disclosed under any subpoena or legal process until after the disclosing party has used reasonable efforts to obtain entry of an appropriate Protective Order limiting the use of such Confidential Information, protecting the Confidential Information from public disclosure and providing for the return or destruction of such Confidential Information upon termination of the legal proceedings. To the extent permitted under applicable law, any party desiring to do so may intervene in any legal proceeding in connection with which any Confidential Information is sought for purposes of protecting such Confidential Information from disclosure or obtaining an appropriate Protective Order.

9. This Agreement will greatly benefit the Parties by facilitating the exchange of Confidential Information and things among the Parties regarding ABF's Proprietary Yeast Strain, MG-FI, and any actual or potential litigation with Biothera. Nevertheless, the Parties understand that they have entered into this Agreement solely for the purposes of facilitating their common interests with respect to ABF's Proprietary Yeast Strain, MG-FI, and any actual or potential litigation with Biothera. The Parties understand that their interests may diverge in the future, and if so, that the Confidential Information made available from one Party to the other will remain confidential and will not be subject to discovery by any Party.

10. The Parties understand that this Agreement does not create any partnership or joint venture between the Parties. Nor does this Agreement in any way impair the Parties' ability to pursue their own interests separately in this or other matters.

11. Nothing in this Agreement will be deemed to novate, modify, abrogate, diminish, increase, amend or otherwise alter the obligations of the Parties, as applicable under any other agreement, including any license agreement or any confidential disclosure agreement, or contract. If any term, provision, or condition of this Agreement is invalid, illegal, or unenforceable in any respect, the validity and enforceability of the remaining terms, provisions, or conditions contained in this Agreement will remain in effect, provided that the parties agree to negotiate in good faith to rewrite a substitute, valid, and enforceable term, provision, or condition which most nearly effects their intent in entering into this Agreement.

12. The terms and conditions of this Agreement will be governed by the laws of the State of Georgia without regard to the conflict or choice of laws of the forum state.

13. This Agreement constitutes the entire Agreement of the Parties with respect to its specific subject matter.

14. This Agreement may be executed in multiple counterparts, and will be binding upon each Party as if each Party executed a single original.

15. The Parties have entered into this Agreement solely for their own benefit; this Agreement is not intended and should not be construed as conferring any rights, benefits or obligations, either directly or indirectly, on any person or entity who is not a Party.

IN WITNESS WHEREOF, the Parties, having caused this Agreement to be executed by their duly authorized representatives, certify that they have full authority to execute this document on behalf of the Parties named herein and that the factual statements made above are true and accurate to the best of their information and belief.

BIOTEC PHARMACON ASA

By:_____

Typed Name:_____

Title:_____

Dated:_____

IMMUNOCORP

By:_____

Typed Name:_____

Title:_____

Dated:_____

ASSOCIATED BRITISH FOODS, PLC

By:_____

Typed Name:_____

Title:_____

Dated:_____

EXHIBIT A

PROPRIETARY YEAST STRAIN CONFIDENTIALITY AGREEMENT

I hereby acknowledge that I am to receive a culture of Associated British Foods, PLC's ("ABF") confidential and proprietary strain of *Saccharomyces cerevisiae* ("ABF's Proprietary Yeast Strain") in connection with Civil No. 05-536 JNE/SRN, Biopolymer Engineering, Inc. v. Immunocorp and Biotec Pharmacon ASA (the "Litigation"), which is pending in the United States District Court for the District of Minnesota. I understand and agree that my testing and examination of ABF's Proprietary Yeast Strain will be solely for purposes related to the Litigation. And because of the highly confidential nature of ABF's Proprietary Yeast Strain, I agree to keep all cultures of ABF's Proprietary Yeast Strain that I receive in a safe place. I will not provide any samples of ABF's Proprietary Yeast Strain to any individual or entity outside of my testing facility, including Biotec Pharmacon, Immunocorp, or Biothera. Additionally, I will instruct any laboratory personnel working under me of the confidential nature of ABF's Proprietary Yeast Strain.

As soon as my examination and evaluation of ABF's Proprietary Yeast Strain is complete, I will immediately destroy all cultures of ABF's Proprietary Yeast Strain in my possession—I will then execute the Certification of Destruction below and provide executed originals of that certificate to counsel for Biotec Pharmacon, Immunocorp, and ABF. Within 30 days after the Litigation is complete, I will destroy all documents generated from my examination and evaluation of ABF's Proprietary Yeast Strain, including any confidential information that I receive regarding the maintenance of ABF's Proprietary Yeast Strain in culture. I further acknowledge that my destruction of ABF's Proprietary Yeast Strain cultures

and any related documents will not relieve me from any of the obligations imposed on me hereunder.

I hereby subject myself to the jurisdiction of the United States District Court for the District of Minnesota for the purpose of any proceedings relating to my obligations hereunder.

Date: _____

Name: _____

Occupation: _____

Business Address: _____

Telephone: _____

CERTIFICATE OF DESTRUCTION

1. My name is _____. I am a resident of _____ _____. I have been employed as a scientific consultant in connection with Civil No. 05-536 JNE/SRN, Biopolymer Engineering, Inc. v. Immunocorp and Biotec Pharmacon ASA.

2. In compliance with the Proprietary Yeast Strain Confidentiality Agreement executed on _____, I certify that I personally destroyed all cultures of ABF's Proprietary Yeast Strain in my possession on the _____ day of _____ 200__.

_____

Sworn to and subscribed before me, this
_____ of _____, 200__.

_____
Notary Public
My Commission Expires:

EXHIBIT B

Attach Copy of Protective Order

EXHIBIT C

UNITED STATES DISTRICT COURT
DISTRICT OF MINNESOTA

Biopolymer Engineering, Inc., d/b/a Biothera

Plaintiff,

v.

Immunocorp, and
Biotec Pharmacon ASA,

Defendants.

Civil No. 05-536 JNE/SRN

AFFIDAVIT of **[Insert Name]**

[Insert Name], being duly sworn, deposes and says:

1. My name is _____. I am a resident of _____ _____. I have been employed by Biopolymer Engineering, d/b/a "Biothera" as a scientific consultant in the above-styled case.

2. On the _____ day of _____, 2006, I received a culture of Associated British Foods, PLC's ("ABF") Proprietary Yeast Strain, which was designated CONFIDENTIAL—COUNSEL AND EXPERTS ONLY under the terms of the Protective Order entered in this matter on August 1, 2005 (Docket Entry No. 33).

3. At the request of counsel for Immunocorp and Biotec Pharmacon and under the terms of the Protective Order, I certify that I personally destroyed all cultures of ABF's Proprietary Yeast Strain in my possession on the _____ day of _____ 200__.

Further affidavit sayeth not.

_____

Sworn to and subscribed before me, this

_____ of _____, 200__.

_____

Notary Public
My Commission Expires:

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